Associations between gene variations and milk composition in New Zealand dairy cattle

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Abstract

Associations between gene variations and milk composition in New Zealand dairy cattle

by

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Milk solids (MS) of various types are an important export commodity for New Zealand (NZ). Of the MS, increased production of milk fat and protein, would increase farm and industry income, and accordingly the production traits of milk volume, fat percentage and protein percentage are included in the National Breeding Objective. This is underpinned by the Breeding Worth (BW) index, which is used to estimate an animal's genetic merit for production. In addition to quantity, the compositions of the milk fat and protein are also important quality determinants for milk processing, milk products, and the nutritional value of milk.

Gene-markers are useful tools for improving animal genetics and breeding. They have become an increasingly useful method for selecting superior dairy cattle, as the valued milk traits are dependent on multiple factors including genetics, breed, diet, and feeding system. In this context, this research set out to ascertain the effect of selected genes on milk traits in NZ pasture-grazed Kiwicross^{TM} cows, and with the overall aim of establishing new gene-markers for cattle breeding.

Five genes were studied, the diacylglycerol acyl-CoA acyltransferase 1 gene (*DGAT1*), the fatty acid binding protein 4 gene (*FABP4*), the stearoyl-CoA desaturase (Δ-9-desaturase) gene (*SCD1*), the perilipin-2 gene (*PLIN2*) and the lipin-1 gene (*LPIN1*). These genes were chosen because they had either been implicated in fatty acid (FA) metabolism in mammary gland cells, or potentially regulated milk fat synthesis. They were screened to find nucleotide sequence variation using a Polymerase Chain Reaction - Single Stranded Conformational Polymorphism (PCR-SSCP) approach, and then a modelling approach was used to ascertain whether the genetic variation (if detected) was associated with gross milk traits (including milk volume, fat percentage and protein percentage), and milk fat composition.

At the gross milk trait level, variation in *DGAT1* and *FABP4* are associated with variation in milk volume, milk fat and milk protein content. Nucleotide sequence variation that has been

reported previously is found in exon 8 of *DGAT1* in the Kiwicross^{TM} cows. If expressed, this variation results in the amino acid substitution p.K232A. The K variant of *DGAT1* (frequency = 61.9% in 395 cows) is found to be associated with the production of less milk volume (KK cows: 22.441 ± 0.526 L/day), but high concentrations of milk fat (KK cows: 5.271 ± 0.067 %) and protein (KK cow: 4.073 ± 0.043 %), than for cows that were A (AA cows: 25.132 ± 0.609 L/day, 4.331 ± 0.077 % and 3.823 ± 0.049 % respectively) ($P < 0.001$).

It has been reported previously that *FABP4* has at least three haplotypes (haplotypes *A*, *B* and *C*), and that these are associated with gross milk traits. Variation in *FABP4* and its association with milk fat composition are therefore investigated. Haplotype *A*, is associated with increased milk C22:0 ($P = 0.001$) and C24:0 FA ($P < 0.001$) levels, and the C10:0, C12:0, and C14:0 FA content and medium chain fatty acid (MCFA) content increased when haplotype *B* was present ($P = 0.012$, $P = 0.009$, $P = 0.005$, and $P = 0.003$ respectively). At the genotype level, *AC* cows produce more C22:0 ($P = 0.021$) and C24:0 ($P = 0.030$) FA, and the AB cows produce more C12:1 ($P = 0.018$) and C14:0 ($P = 0.010$) FA.

In respect of milk fat composition, variation in *DGAT1* and *SCD1* are associated with variation in milk FAs. Cows with the *DGAT1* p.232 K variant, produce more saturated FAs ($P < 0.001$) but less branched and unsaturated FAs ($P < 0.001$). The p.232 homozygous AA cows produce more ($P < 0.001$) CLA (1.070 ± 0.054 g/100 g) and C18:3 *cis*-9, 12, 15 FA (0.830 ± 0.021 g/100 g), but less C16:0 FA (35.739 ± 0.534 g/100 g) than the KK cows (0.864 ± 0.046 g/100 g, 0.751 ± 0.018 g/100 g and 38.437 ± 0.461 g/100 g respectively).

For the SCD1 gene (*SCD1*), three genetic variations in exon 5 (c.702A>G, c.762T>C and c.878C>T) were found and the c.878C>T substitution would result in amino acid change p.A293V. One variation in intron 5 (c.880 + 105A>G) and four variations in the 3'UTR (c.*1783A>G, c.*1883C>T, c.*1984G>A and c.*2066T/C /G) were also found. The c.*1783A>G and c.*2066T/C/G substitutions produced three nucleotide sequence variants (*a*, *b* and *c*). There was linkage between the exon 5 variation and the 3'UTR variation, such that the sequence that would encode valine at position 293 of SCD1 is linked to 3'UTR variant *a*, and the sequence that would encode alanine, is linked to variants *b* and *c*. The VV cows produced less C10:1, C12:1 and C14:1 FA, but more C16:1 and C18:2 FA than the AA cows ($P < 0.001$). The presence of *c* is associated with decreased levels of C16:1 ($P < 0.001$), C17:1 ($P = 0.001$), C18:2 *cis*-9, *trans*-13 ($P = 0.045$), C18:2 *cis*-9, *trans*-12 ($P = 0.018$) FA and C16:1 FA index ($P < 0.001$). The presence of *b* is associated with increased levels of C13:0 *iso* FAs ($P < 0.001$), MUFA ($P = 0.002$), and C12:1 ($P < 0.001$).

Variation in the 3'UTR of *PLIN2* (c.*302T>C), which produce two nucleotide sequence variants (A_5 and B_5) was described. The B_5B_5 homozygous cows produced less palmitic acid (C16:0) (P = 0.048), but more medium chain fatty acids, than the A_5A_5 cows (P = 0.033).

Overall, this study identified *DGAT1* and *FABP4* as good candidate genes for predicting gross milk trait variation in Kiwicross^{TM} cows. Furthermore, *DGAT1*, *FABP4*, *SCD1* and *PLIN2* might be useful for predicting variation in milk fat composition.

Variation was not found in the regions (exon 16, exon 17, part of intron 16 and part of intron 17) of *LIPN1* that were investigated.

To further this research, more dairy cattle from different dairy production systems will need to be studied to confirm that these genes might be useful markers for gross milk traits and milk fat composition.

Keywords: milk traits, dairy cattle, fatty acids, *DGAT1*, *FABP4*, *SCD1*, *PLIN2*, *LPIN1*.

Publications arising from this study

Yunhai Li, Huitong Zhou, Long Cheng, Grant R. Edwards & Jonathan G. H. Hickford (2019). Effect of *DGAT1* variant (K232A) on milk traits and milk fat composition in outdoor pasture-grazed dairy cattle. *New Zealand Journal of Agricultural Research* (In press)

Yunhai Li, Huitong Zhou, Long Cheng, Marian Hodges, Rosy Tung, Jenny Zhao, Grant R. Edwards & Jonathan G. H. Hickford (2020). Effects of *FABP4* variation on milk fatty-acid composition in dairy cattle grazed on pasture in late lactation. *Journal of Dairy Research*, *87*(1), 32-36.

Yunhai Li, Huitong Zhou, Long Cheng, Grant R. Edwards & Jonathan G. H. Hickford (2020). Variation in the bovine SCD1 gene affects milk fatty-acid composition in outdoor pasture-grazed dairy cattle. *Journal* (submitted).

Yunhai Li, Huitong Zhou, Long Cheng, Grant R. Edwards & Jonathan G. H. Hickford (2020). Effects of *PLIN2* variation on milk fatty-acid composition in dairy cattle grazed on pasture in late lactation. *Journal* (submitted).

Ishaku Haruna; Yunhai Li; Jane Ekegbu; Hamed Amirpour Najafabadi; Huitong Zhou & Jon Hickford. (2020). Associations between bovine MSTN variants and milk production traits in New Zealand Holstein-Friesian × Jersey cross cows. *Journal* (submitted).

Haruna, Ishaku; Li, Yunhai; Zhou, Huitong & Hickford, Jon. (2020). Effects of bovine leptin gene variation on milk traits in New Zealand Holstein-Friesian × Jersey-cross dairy cows. *Journal* (submitted).

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Abbreviations

$	Dollar
%	percentage
ºC	degree Celsius
µL	microlitre
µm	micrometre
A	adenine
ABCG2	adenosine triphosphate-binding cassette efflux transporter 2
ACAT	acylCoA: cholesterol acyltransferase
ADFP	adipose differentiation-related protein
AFLP	Amplified Fragment Length Polymorphism
AP-1	activation protein-1
Bp	base pair
BW	Breeding Worth
C	cytosine
CE	cholesterol esters
CLA	conjugated linoleic acid
Cm	centimetre
DGAT1	diacylglycerol acyl-CoA acyltransferase 1
DM	dry matter
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
FABP4	fatty acid binding protein 4
FSE1	fat-specific enhancer 1
G	guanine
GC	Gas Chromatography
h	hour
HF	Holstein-Friesian
INDEL	insertion-deletion
JE	Jersey
Kg	kilogram
LIPIN1	lipin 1
LCFA	long chain fatty acid

MCFA	medium chain fatty acid
MEF-2	myocyte-specific enhancer-binding factor 2
mg	milligram
mL	millilitre
mM	millimolar
mm	millimetre
MS	milk solid
MUFA	monounsaturated fatty acid
NCBI	National Centre for Biotechnology Information
NEB	negative energy balance
ng/mL	nanograms per millilitre
NZ	New Zealand
PCR	Polymerase Chain Reaction
PLIN2	Perilipin-2
pH	Potential of Hydrogen
PPARα	peroxisome proliferator-activated receptor α
PPARGC1A	peroxisome proliferator-activated receptor-gamma, coactivator 1α
PUFA	polyunsaturated fatty acid
QTL	quantitative trait loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SCD1	stearoyl-CoA desaturase
SCFA	short chain fatty acid
SNP	Single Nucleotide Polymorphism
SSCP	Single Stranded Conformation Polymorphism
T	thymine
TAG	triacylglycerol
Taq	*Thermus aquaticus*
TBE	tris-borate-EDTA
TE	Tris-EDTA
Tris	Tris (hydroxylethyl) aminomethane
U	unit
UFA	unsaturated fatty acid
VNTR	variable number of tandem repeats

Amino Acid	Three-letter Abbreviation	One-letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1. Introduction

New Zealand (NZ) is a major exporter of dairy products. Dairy production contributed approximately 23% of NZ export revenue in 2002 (Clark *et al.*, 2007), this growing to 25% in 2012 (Foote *et al.*, 2015). Given this economic value, the population of cows in NZ has also increased. The statistics (New Zealand Dairy Statistics 2018-2019) produced by DairyNZ and the Livestock Improvement Corporation Ltd (LIC, Hamilton, NZ) for the 2018-2019 season (DairyNZ, 2019), revealed that the numbers of dairy cattle increased from 2.1 million in 1975 to nearly 4.9 million in 2019. The population of cows over this period reached a peak of 5.0 million in 2014. In concert with the increasing cow population, the land area for dairy farms in 2016 (1.7 million hectares) was about 1.5 times higher than in 1992 (1.1 million hectares) (DairyNZ, 2017).

With a small domestic market for whole milk and milk products, the export of processed and manufactured dairy products to overseas markets drives export trade. Given that many of the exported products are rendered or processed, the production of milk fat and protein are more important than the production of milk volume for NZ dairy farmers. Their payment for milk supplied is therefore typically based on milk solid (MS) content.

Nearly all cows in NZ are farmed in outdoor pasture-based systems, with varying levels of feed supplementation. While production can be, and is being improved by improving cow nutrition, the improvement of herd genetics through breeding is another way of improving dairy industry performance.

Breeding for dairy production in NZ is driven strongly by a National Breeding Objective and a breeding system that is administered by the independent body NZ Animal Evaluations Ltd (NZAEL), a subsidiary of DairyNZ (www.dairynz.co.nz/animal/animal-evaluation/). The system produces Breeding Worth (BW) index, and it includes estimates of an animal's genetic merit (using estimated breeding values eBVs) for eight key traits that are of value to the NZ dairy industry. These traits are: milk fat production (measured as the percentage fat in milk), milk protein production (measured as the percentage of protein in milk), the overall milk volume, a cow's live-weight, somatic cell score, fertility, body condition score and residual survival.

Historically, about 100 kg of milk fat could be made by a single Holstein-Friesian cow in one year, and the average milk fat composition in fresh milk was about 3.4% (Gowen, 1924).

Following the introduction of the National Breeding Objective a single cow can now produce in excess of 214 (2016/2017 season) and 207 kg (2017/2018 season) of milk fat in a year (DairyNZ, 2018b). This milk fat is composed of different lipids: including phospholipids, cholesterol, triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids (FFA), monoacylglycerol (MAG) and cholesteryl ester. Of these lipids, TAG is the most common component (approximately 95%) of milk fat (Jensen *et al.*, 1995; Stelwagen, 2011). However, there are more than 400 different FAs in milk, and variation in the milk FA content can change the processing performance, end-use and nutritional value of milk.

To date, most research into milk fat and milk fatty acid content has been undertaken with the so-called 'pure-bred' Jersey and Holstein-Friesian cows that historically dominated the national dairy herd. In 2018 DairyNZ (2018b) reported that the Kiwicross^{TM} cows (Holstein-Friesian × Jersey-cross cows of varying breed proportion) were the predominant 'breed' at 47.8% of the dairy cow population. As there has been less research into these cross-bred cows, little is known of how milk fatty acid content may have changed with their introduction, although one might expect it to lie somewhere within the range defined by the parent breeds. Additionally, little is known of the bovine genes that may control both gross milk traits and milk FA composition. Accordingly, the research described in this thesis (Figure 1.1) aims to identify or better understand variation in several genes that may be associated with variation in gross milk traits and milk fat composition in pasture-grazed Kiwicross^{TM} cows.

The genes chosen for investigation included the diacylglycerol acyl-CoA acyltransferase 1 gene (*DGAT1*), the fatty acid binding protein 4 gene (*FABP4*), the stearoyl-CoA desaturase (Δ-9-desaturase) gene (*SCD1*), the perilipin-2 gene (*PLIN2*) and the lipin-1 gene (*LPIN1*). These genes have previously been implicated as having a role in milk fatty acid synthesis (Winter *et al.*, 2002), uptake (Nafikov *et al.*, 2013), desaturation (Garnsworthy *et al.*, 2010), release (Imai *et al.*, 2007; Imamura *et al.*, 2002; Magra *et al.*, 2006) and accumulation (Phan *et al.*, 2004) respectively.

Polymerase Chain Reaction - Single-Stranded Conformational Polymorphism (PCR-SSCP) analyses were used to detect variation in key functional regions of the five genes, and if present, then DNA sequencing was used to characterise the genetic variation detected. Association analyses were then undertaken to ascertain if the variation in the five genes could reliably predict variation in both gross milk traits and milk FA composition.

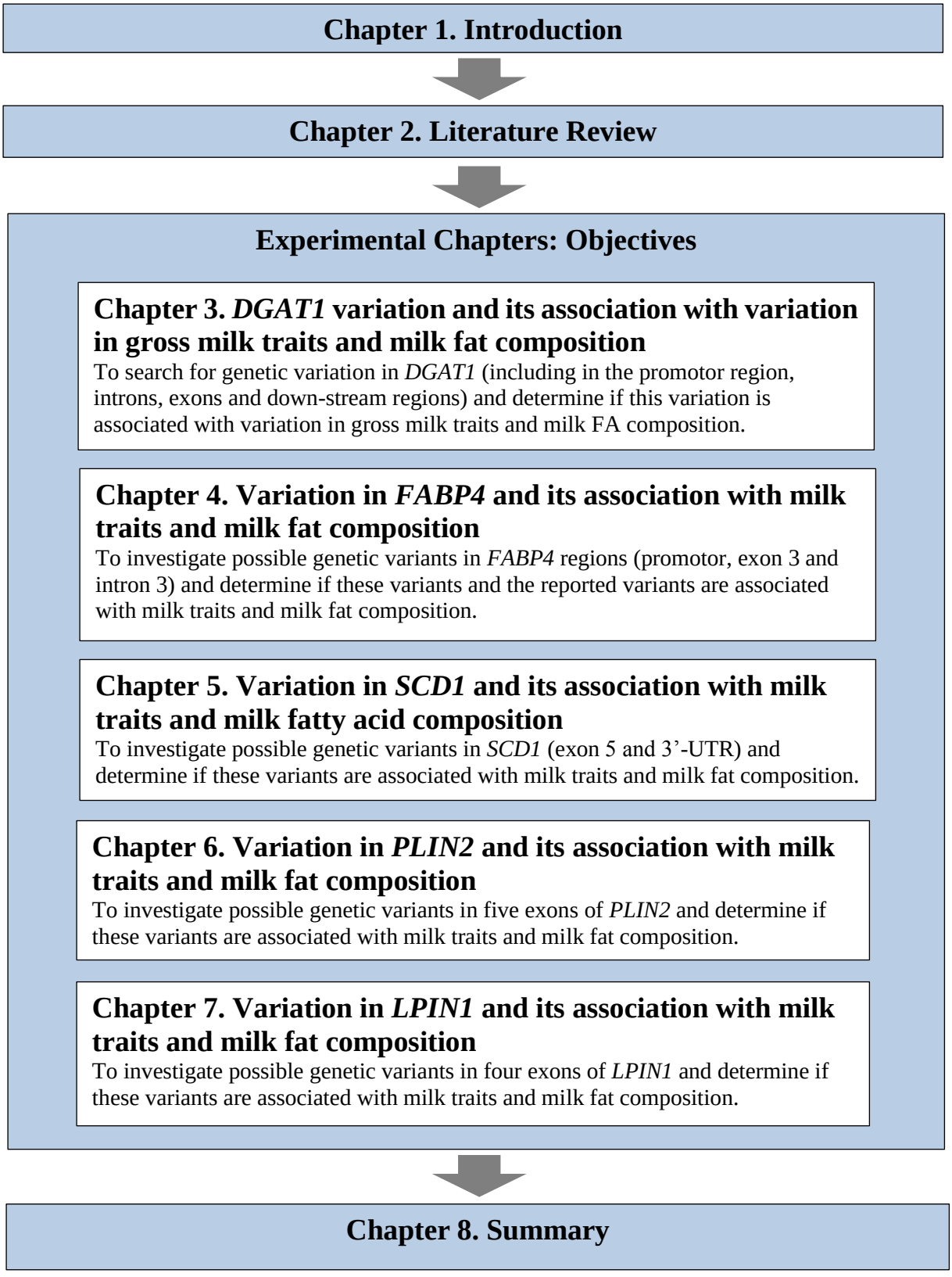


Figure 1. 1 Outline of the thesis chapters.

Chapter 2. Literature Review

2.1. The role of milk in the New Zealand dairy industry

The milk of dairy cows is of nutritional value to humans, and it is therefore sought after globally. New Zealand produces considerably more milk than needed for domestic consumption, so a thriving export industry has been developed, and milk production contributes a large amount of value to NZ's export economy. In 2010, data from the NZ Institute of Economic Research (NZIER) revealed that the dairy industry in NZ contributed about 2.8% of GDP (approx. NZ$5.0 billion) to the national economy and it provided 26% of NZ's total export goods (Chris, 2010). In 2016, the industry had grown and the NZIER revealed that the dairy industry in NZ contributed about 3.5% of GDP, with an increased value of approximately NZ$7.8 billion (John, 2017).

Most of the milk produced in NZ is processed and manufactured into products that are exported to overseas markets, and only a small proportion of the milk is consumed domestically. Given that milk fat and protein are the main raw materials for processing into the various products, then payment to dairy farmers in NZ is based primarily on MS production. The New Zealand Dairy Statistics 2017-2018 (DairyNZ, 2018b) have summarised NZ milk production from the 1983/84 season to the 2017/18 season (Figure 2.1) and revealed that the production of processed MS increased rapidly from 564 million kg in 1983/84 to a peak of 1890 kg in 2014/15. There was a slight decrease in MS production in the 2015/16, 2016/17 and 2017/18 season, but the average MS production per cow has increased since 1975/76, reaching 381 kg per cow in the 2016/17 season (Figure 2.2). Consistent with this increase in total MS production per cow has been increased production of milk protein and milk fat, with more fat typically being produced than protein.

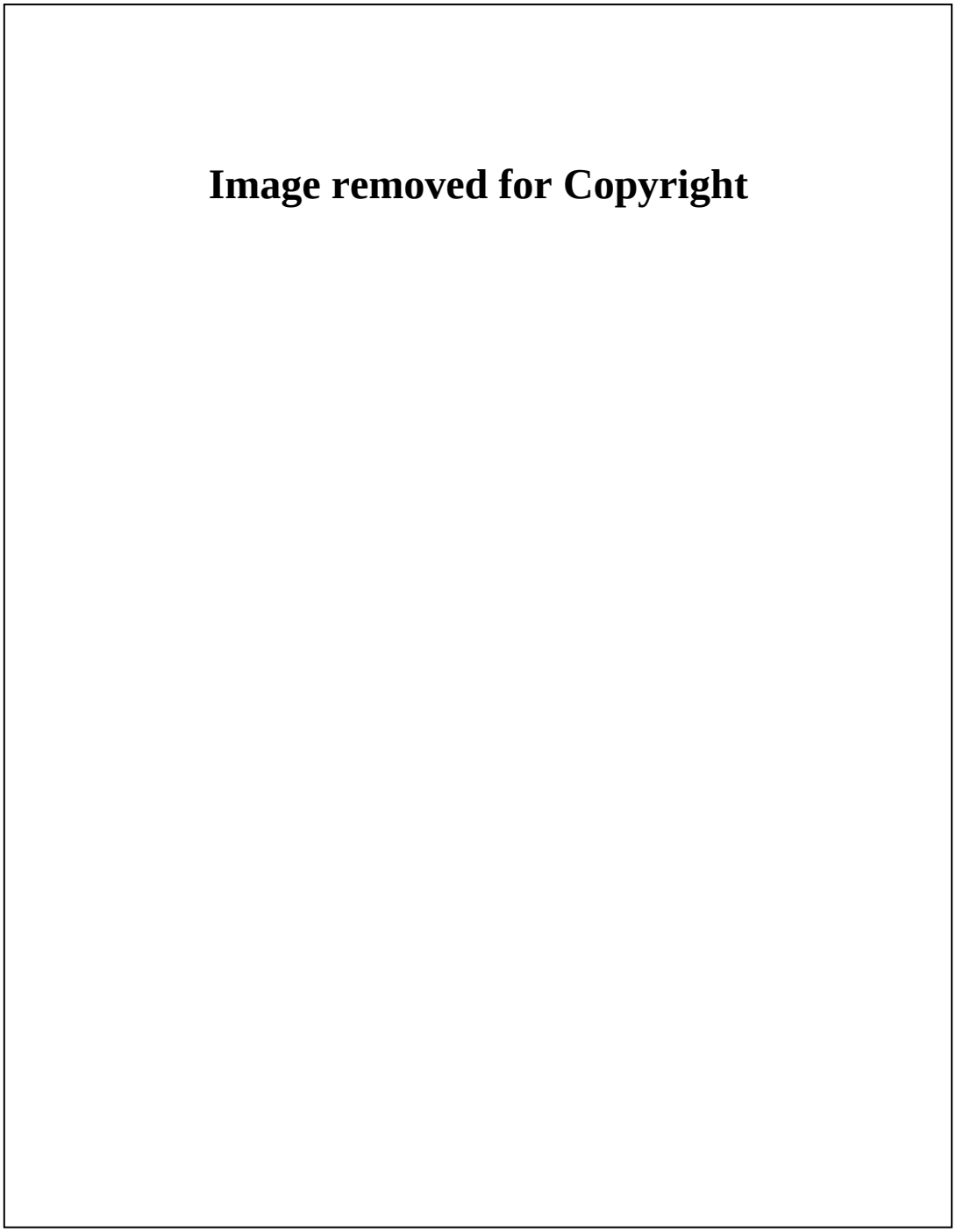


Figure 2. 1 Summary of milk production statistics for 35 season (DairyNZ, 2018b).

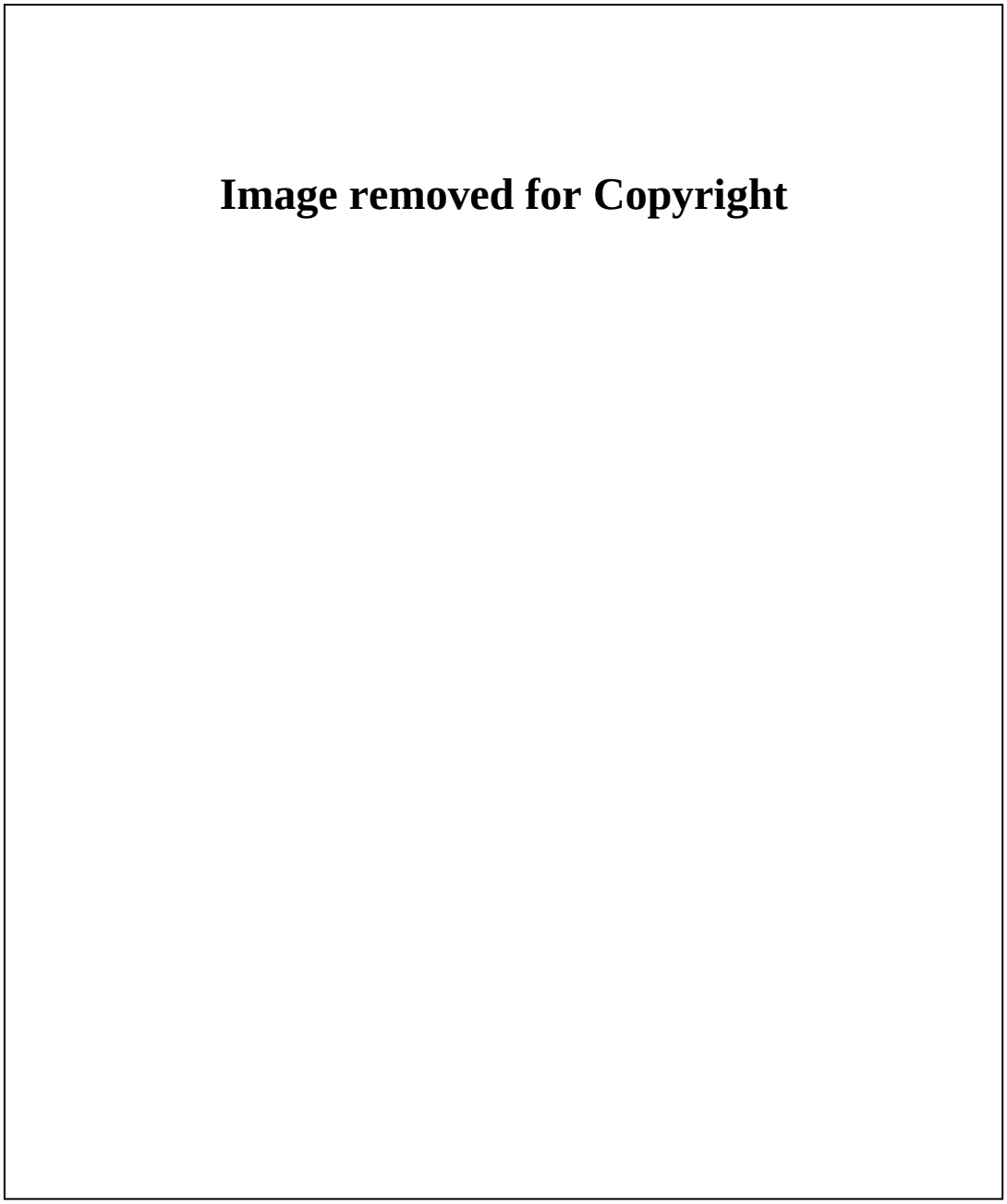


Figure 2. 2 Summary of herd production since 1975/76 (DairyNZ, 2018b). The main season on season trend one observes is increased kilograms of milk fat produced per cow, kilograms of protein produced per cow, and kilograms of MS produced per cow; although at times minor fluctuations are observed, these likely reflecting climatic effects.

2.2. Factors that can affect gross milk traits in NZ dairy cows

The factors that can affect gross milk traits, such as the content of protein and fat in milk, are numerous and include environmental factors (e.g. climate variation), management factors (e.g. differences in feeding systems, nutrient availability and diet), and animal factors (e.g. differences in breed, stage of lactation, cow age, and disease occurrence). What-is-more, each factor can also potentially affect more than one trait. As a consequence of this, any analyses of the factors that might affect milk traits, need to be a multivariate analysis.

For example, Stelwagen (2011) identified that milk fat production could be affected by breed, lactation stage, animal body condition, diet and environment; and Mele *et al.* (2016) suggested that the effects of dairy production system, feeding regime, herd, cow parity and stage of lactation were considerable, when analysing milk FA composition. In response to this, Macciotta *et al.* (2004) developed a multivariate approach, based on various environment and genetic factors, to predict a cow's lactation curve.

Cows in NZ are typically grazing year-round on pasture, but with some use of various types of feed supplementation, especially in the winter period when cows are 'dried-off' and not milked. One well studied factor that influences milk production is the quality of pasture, and its seasonal growth pattern. Given that pasture growth typically peaks in the spring, the NZ dairy system is designed around cows annually producing a calf in the early spring period, and so as to capture maximal value out of that growing pasture. The peak of pasture growth changes to some degree as a consequence of latitude and the associated temperature variation that occurs North to South in NZ, but it essentially means that all cows are approximately at the same lactation stage. The effect of lactation stage and animal body condition on milk traits cannot therefore be ignored.

2.2.1. The effect of diet on milk fat production and composition

Dairy pasture in NZ is typically based on perennial ryegrass (Lolium perenne) and white clover (Trifolium repens) (James et al., 1999). This pasture provides a low-cost source of nutrients (Penno et al., 2007), and requires minimal use of feed supplements.

In the 2016-2107 season the DairyNZ Economic Survey (DairyNZ, 2018a) reported that pasture (including hay and silage/baleage) accounted for approximately 82 % of total feed dry matter (DM) intake, with palm kernel extract (PKE) accounting for 6%, and fodder beet 4%.

While new feeds and feeding systems might be adopted in the future, the main component of the diet of NZ dairy cows will still predominantly be pasture. Accordingly, any analysis of gross milk traits and milk fatty acid composition in NZ needs to be contextualised in respect of this predominantly pasture-based production system, and not a total mixed ration (TMR), or any other feeding approach.

From a human health perspective pasture-based production system are of interest. Specifically, it has been suggested that these diets lead to higher levels of polyunsaturated fatty acids (PUFA) and conjugated linoleic acids (CLA) in the milk (Chilliard et al., 2001; Dewhurst et al., 2006).

2.2.2. The effect of lactation stage on milk fat production and composition

The most distinctive feature of the lactation cycle in NZ dairy cows is that all cows are at a very similar lactation stage. The changes in gross milk traits over a lactation have been described graphically in Figure 2.3 (Roche *et al.* 2006). Consolidated effects of strain (Figure 2.3a) or concentrate supplementation (Figure 2.3b) on milk traits were found over DIM = 50 – 300. However, these effects could be interfered with when cows in the early stages of lactation (DIM < 50). The effect of lactation stages on milk traits were mainly because of the change of cow's body condition.

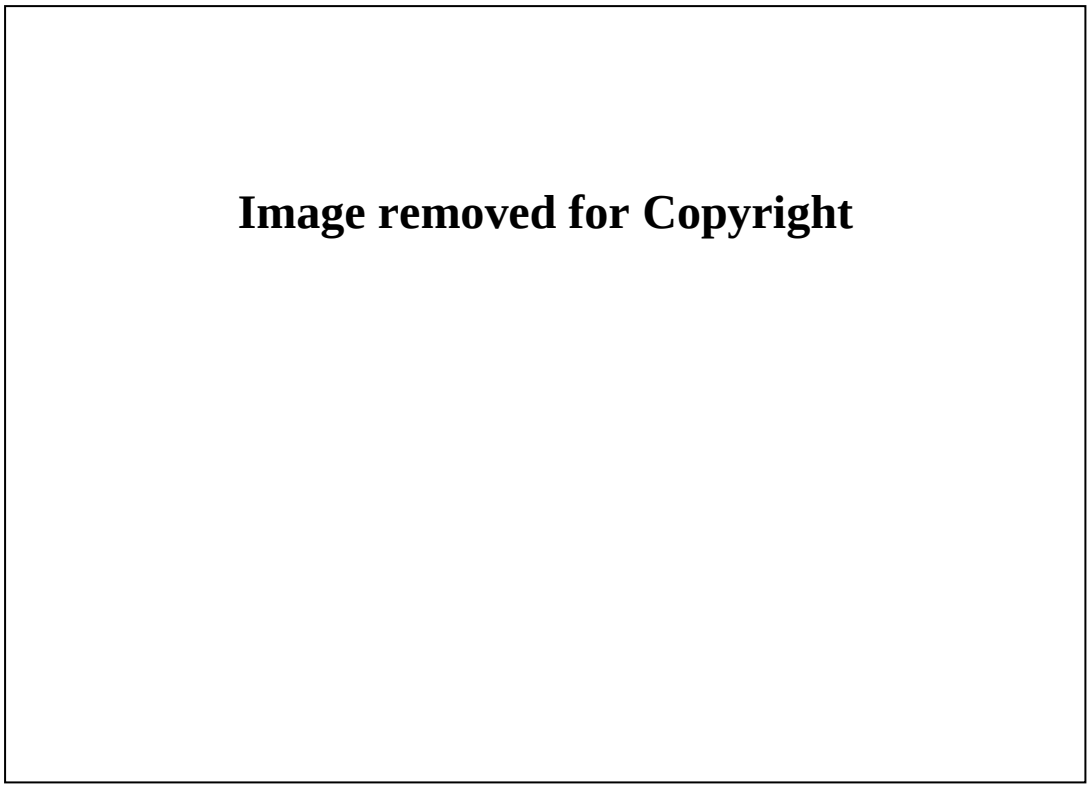


Figure 2. 3 Average lactation profiles for milk yield, fat and protein percentage. (a) Effect of strain of Holstein-Friesian dairy cow on the lactation profile for milk yield. Production from North American Holstein-Friesian dairy cows (■) versus New Zealand Holstein-Friesian dairy cows (♦). (b) Effect of level of concentrate supplementation in cows receiving 0 (♦), 3 (■), or 6 (▲) kg DM of a concentrate pellet daily, throughout lactation (Roche *et al.*, 2006).

One feature of early lactation is that cows are in negative energy balance (Strucken *et al.*, 2015). Figure 2.4 illustrates how dietary energy intake is unable to meet the demands of high milk production in approximately the first 60 days of lactation (the difference between the purple line and the blue line in the figure). Accordingly, the mobilisation of body energy stores or reserves has to occur to balance the deficit between dietary energy intake, and energy expenditure on maintenance and milk production (Bauman *et al.*, 1980). As a consequence of the cow's body fat being mobilised to meet this energy deficit, other biological pathways are affected, and this changes milk composition.

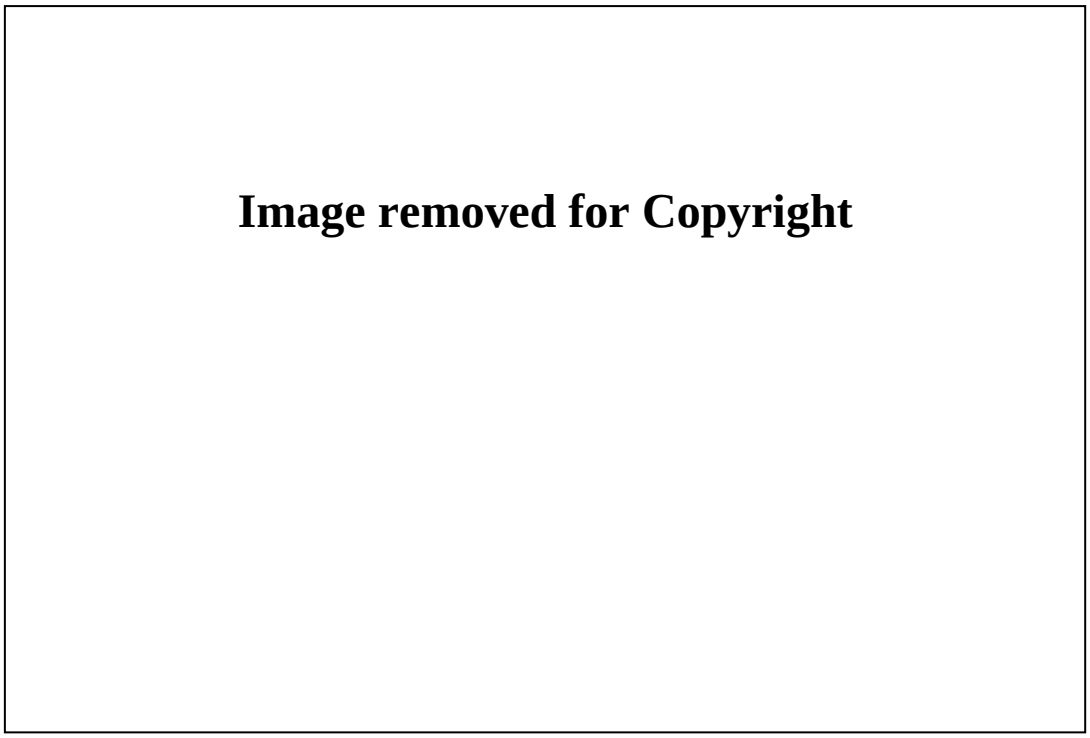


Figure 2. 4 Energy supply and requirements for milk production and maintenance during the lactation cycle (Strucken et al., 2015). The blue line represents the energy demand for lactation and maintenance, the purple line represents energy supply from feed, and the period of negative energy balance is indicated by red cross-hatching. This is the period when cows typically lose condition.

As the energy supply for milk fat production changes from body fat mobilisation (in early lactation), to food intake and *de novo* synthesis of FA (in mid and late lactation stages), milk fat % continues to increase (Figure 2.3). What is more, milk fat composition changes, as the ratio of *de novo* or 'synthesised' FA, to 'imported' fat changes (Figure 2.5).

Overall, cows in negative energy balance produce a greater proportion of saturated FA (SFA - mainly C16:0 and C18:0 FA). When the negative energy balance period ends, *de novo* synthesis of FA becomes the main source of milk fat and typically the saturation ratio of milk FA changes after the 60th DIM. Stoop *et al.* (2009) also described how most milk FA levels (except the C5, C15, branched and CLA *trans*-10, *cis*-12 FA levels), changed in the middle and late stages of lactation.

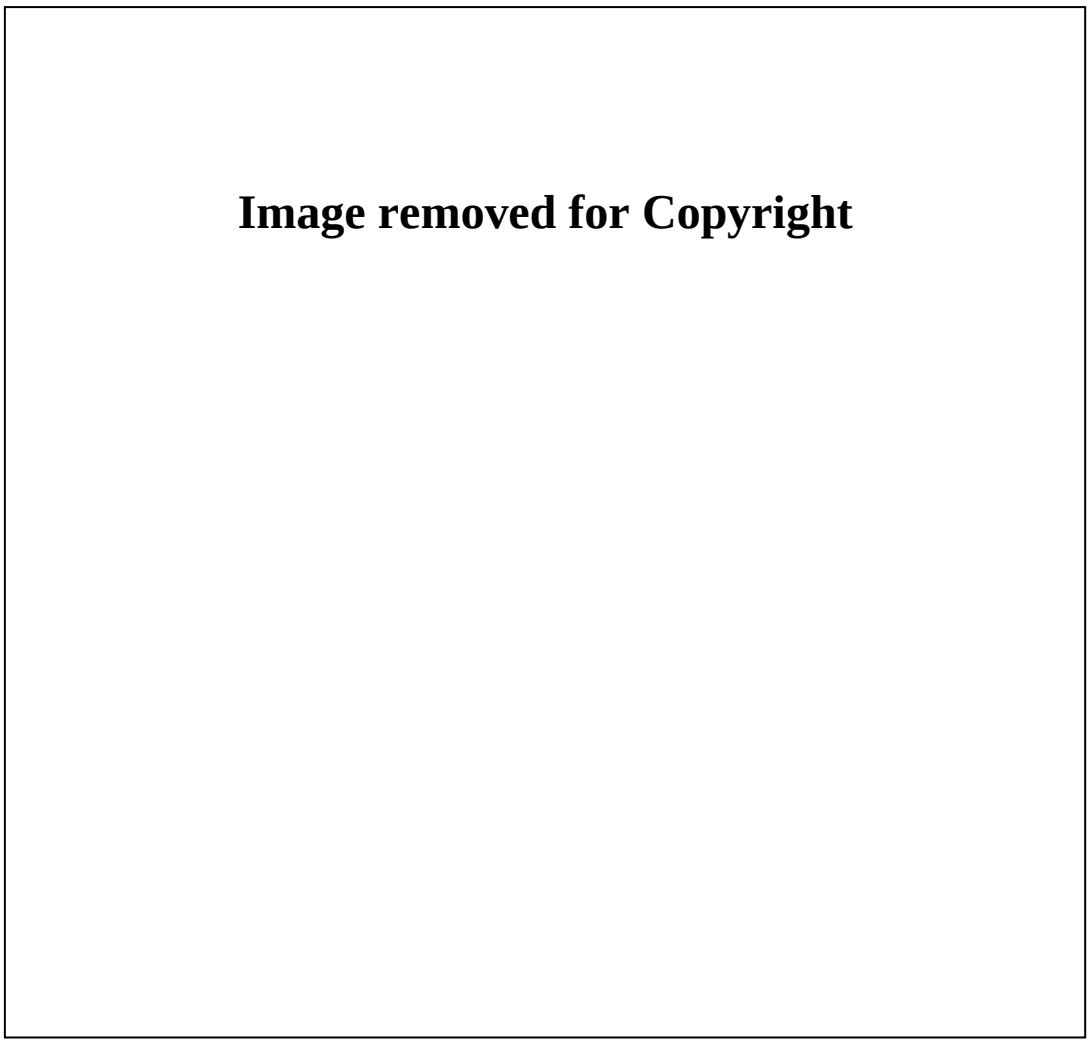
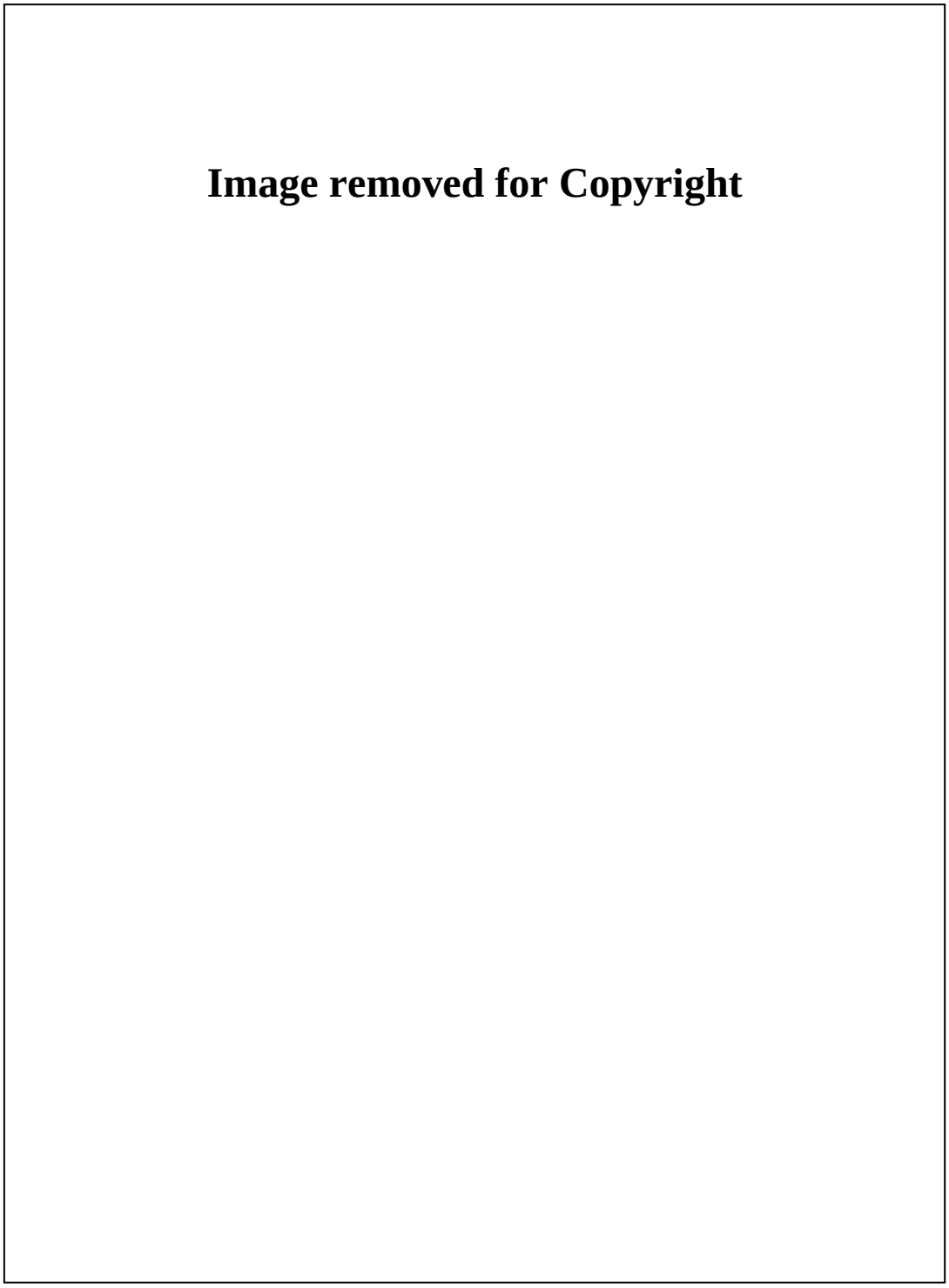


Figure 2. 5 De novo FA synthesis (Synthesized) vs imported FA (Imported) (Top panel) and Δ9 desaturase indexes during lactation (Bottom panel). Synthesized = FA with 4-14 carbons except 11:1; pooled SEM = 0.34. Imported from blood = FA with carbon chain >16 plus 15:0 and 11:1; pooled SEM = 0.31. Synthesized/Imported, pooled SEM = 0.10. Pooled SEM for Δ9 activity on 14:0, 16:0, 18:0, and C18:1 trans-11 was 0.008, 0.007, 0.02, and 0.06, respectively. There is a statistically significant effect of time ($P < 0.05$) for all measurements except Synthesized FA levels ($P = 0.24$) and Δ9 activity on 18:0 ($P = 0.77$) (Bionaz et al., 2008b).

Another notable feature of the New Zealand dairy production system is that while most cows are in a pasture-based feeding system, the nature of the pasture is changing, this being driven by the need for greater production efficiency and to reduce nutrient losses. For example, Cheng *et al.* (2017) investigated the effects of a diets that include plantain and perennial ryegrass-white clover pasture on the reduction of nitrogen excretion in urine, while Fleming *et al.* (2018) supplied sixty New Zealand Friesian x Jersey cows (age = 3.6 ± 0.12 years; DIM =

85 ± 4.8 days) a perennial ryegrass-based diet with fodder beet, that was revealed to change milk fat percentage and composition, but not the milk yield and MS levels (Table 2.1).

Table 2. 1 Change in yield of milk and milk constituents and milk fatty acid (FA) composition of cows fed either herbage only (H) or herbage and 4 kg DM of harvested fodder beet (FBB) (Fleming *et al.*, 2018).



Means in the same row with different subscripts are significantly different.

2.2.3. Breed selection for increasing milk solid (MS) production

Breeding for dairy production in NZ is strongly influenced by the use of a breeding index system (www.dairynz.co.nz/animal/animal-evaluation/) that is administered by NZAEL, a subsidiary of the industry-good body, DairyNZ. This is known as the Breeding Worth (BW) index, and it includes various estimates of an animal's genetic merit (based on the calculation of eBVs) for eight traits that are of value to the NZ dairy industry. The effective emphasis on the eight traits within Breeding Worth are: milk volume (13%), milk fat production (24%), milk protein production (17%), cow live-weight (11%), somatic cell score (6%), fertility (13%), body condition score (7%) and residual survival (9%) (DairyNZ, 2019). Each trait has a unique assessment regime. The BW index is used to select dairy cattle that are predominantly of three types, Jersey (J), Holstein-Friesian (HF) and Holstein-Friesian × Jersey (HF×J)-cross (or Kiwicross^{TM}) cows.

The first Dutch Friesian cattle were imported by Canterbury's John Grigg in 1884. These black and white cattle quickly gained popularity in the North Island, but it wasn't until 1910 that their breeding and importation was taken up seriously. Early endeavours by breeders to keep accurate pedigrees of these animals resulted in the New Zealand Holstein-Friesian Association being established in 1910.

The first pedigree Jersey cattle were two cows and a bull brought to Whanganui by Thomas Syers in 1862. Their cows are typically smaller than Holstein-Friesian cows. From 1886 to the present day, the purebred Jersey has been registered in a New Zealand herd book, and this is administered by the Purebred Jersey Breed Society of New Zealand.

Harris *et al.* (2001) have described how the NZ Holstein-Friesian population developed and became popular, but their daughters were heavier, less fertile and had lower survival rates (Harris *et al.*, 2001). As a consequence, the cross-breeding of cattle breeds became one of the alternatives for improving dairy production.

Lopez-Villalobos *et al.* (1996) demonstrated the economic superiority of cross-bred HF×J cows in NZ, and a genetic evaluation suitable for these cross-bred cattle started in the same year (Harris *et al.*, 1996). In 2002, one-third of the NZ dairy cow population was crossbred, mostly HF×J, and the proportion of HF×J-cross cattle increased to 48.5% in the 2018/19 season (Figure 2.6). The name Kiwicross^{TM} was coined to identify these cows.

Figure 2. 6 Breed/cross breakdown for dairy cows in NZ in the 2018/19 season (DairyNZ, 2019).

After breeding for many years, the cows of NZ have become very effective producers of milk solids (Figure 2.2). In the 2016/2017 season, the average NZ dairy cow produced 214 kg of milk fat, and 167 kg of milk protein, or alternatively 603 kg of milk fat per hectare of pasture and 460 kg of milk protein per hectare. This is not wholly attributable to genetics or breeding, and pasture quality and quantity have also improved over time.

Not only has total MS production increased, but the percentage of milk fat in whole milk has also increased. Historically, about 100 kg of milk fat could be made by a single Holstein-Friesian cow in one year, and the average milk fat composition in fresh milk was about 3.4% (Gowen, 1924) , but in the 1997/98 season the fat content of milk averaged 4.67 %, increasing to 4.77 % in the 2017/18 season (DairyNZ, 2018b).

Woodford *et al.* (1986) have described how, both within and between breed, cows of different genetic background can have differences in milk fat production, while Bobe *et al.* (2008) described the genetic variability of milk fatty acid levels in Holstein cows in the United States. Bobe *et al.* (2008) estimates infer genetic variability in milk yield, milk concentration and fatty acids levels (Table 2.2). Accordingly, milk FA content can be altered by selective breeding, with the effect being from both variation in the total fat yield and proportions of individual FAs (particular in the medium- and long-chain FAs).

Table 2. 2 Genetic parameter estimates (mean ± SE) for milk yield, milk concentration, and relative proportion of milk fatty acids (FA) in US Holstein cows[1] (Bobe *et al.*, 2008).

[1] 592 daily samples from 233 cows of 53 sires.
[2] Units of measure were g/d, except for milk measured in kg/d.
[*] $P \leqslant 0.05$; [**] $P \leqslant 0.01$.

The reasons for attempting to change milk FA yield and composition are likely many, but they may include being able to produce milks with different processing performance, and/or with health benefits. For example, long chain saturated fatty acids typically produce 'harder' fats, while the health benefits of the omega-3 FA have been extensively discussed (see https://ods.od.nih.gov/factsheets/Omega3FattyAcids-Consumer/ for a credible and comprehensive discussion).

While probably not impossible, it would nevertheless be a time-consuming and costly exercise to select cows that had a unique and heritable milk fat composition by traditional methods, hence gene technology may provide a more effective way of selecting superior cattle with specific milk fat compositions. A variety of gene technology approaches could be employed, but a frequently used approach is to assess the effect of known, and at times well-characterised genes, on traits of interest.

In the context of gross milk traits (including milk fat content) and milk FA composition, then the selection of genes that are thought or have been revealed to be involved in fatty acid synthesis, transport, storage and breakdown, would be a useful starting point.

2.3. Genes that may affect milk fat production and FA composition in cows

There are many different physiological and biochemical ‘clues’ that can indicate or suggest the genes (or gene activities) that might contribute to variation in milk fat production and FA composition. These genes can be active in different metabolic pathways, including pathways involved in the regulation of feed intake, energy availability, growth, cell proliferation, apoptosis, and triglyceride synthesis (Figure 2.7).

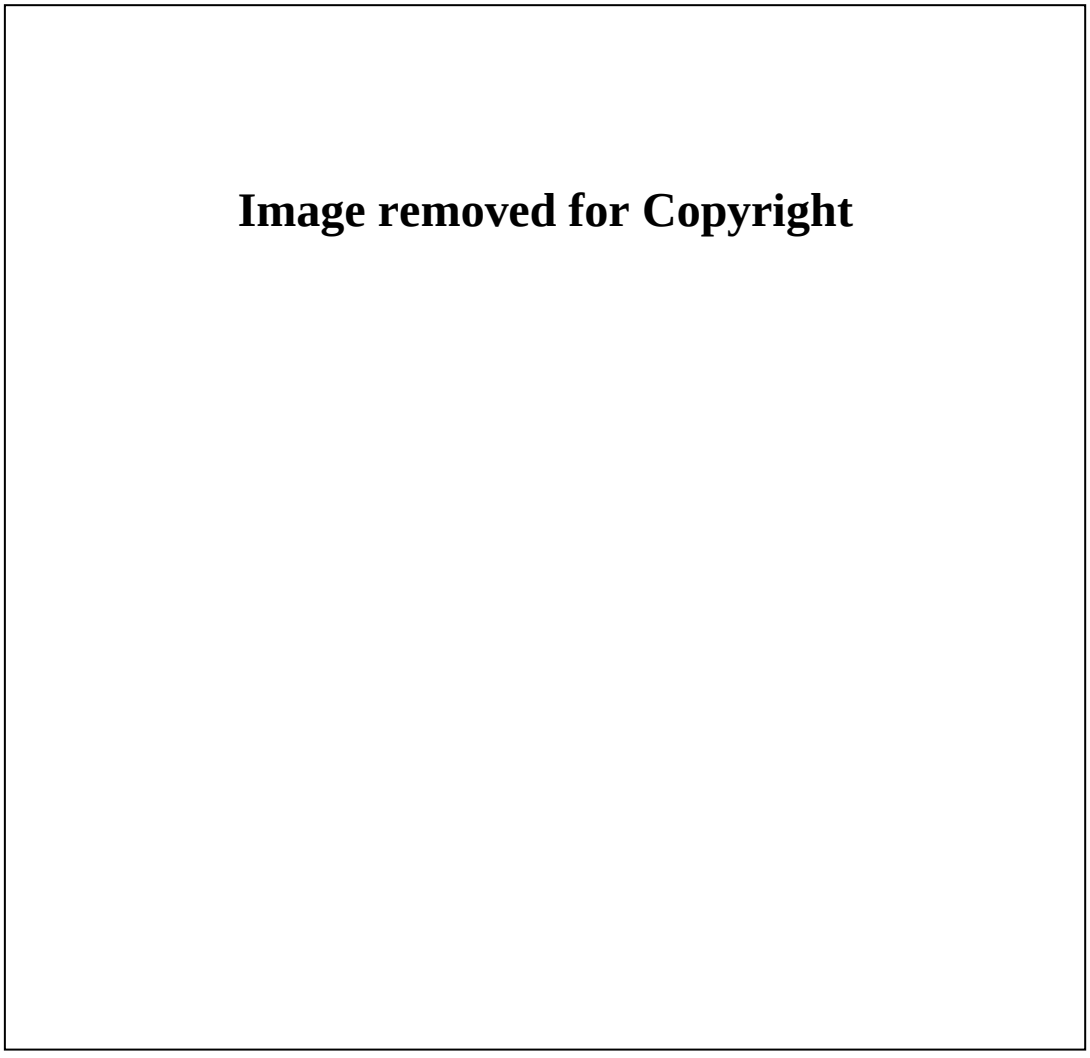


Figure 2. 7 Simplified pathways describing some genes involved in milk production. The rectangular green boxes represent different genes and the oval shapes describe some of the various pathways that the genes are involved in (Strucken *et al.*, 2015).

Milk fat composition can be described in terms of types of lipid, or individual FA levels. The milk fat lipids include phospholipids, cholesterol, TAG, DAG, MAG, FFAs, and cholesteryl ester. While the main component of milk lipid is TAG, and for cattle this constitutes more than 95% of total milk lipid (Jensen *et al.*, 1995; Stelwagen, 2011). There are more than 400 kinds of FA found in cows' milk. Nearly half of these milk FAs are synthesised *de novo* in the mammary gland cells of ruminants. These FAs are mainly short, middle chain FA (i.e. C4 to C14), although C16 FA is also produced. The remaining milk FA are transported from blood to the mammary gland, and they are predominantly long chain fatty acid (LCFA), although C16 FAs are also sourced from the blood (Duchacek *et al.*, 2012). Bionaz *et al.* (2008b) described some of the gene networks and candidate genes involved in bovine milk fat synthesis (Figure 2.8). The products of these candidate genes could be classified into categories including proteins involved in fatty acid transport, FA synthesis, FA desaturation and release, and the genes include the fatty acid binding protein 4 gene (*FABP4*), the diacylglycerol O-acyltransferase gene (*DGAT1*), the stearoyl-CoA desaturase gene (*SCD1*), and the perilipin-2 gene (*PLIN2* also known as *ADFP*).

Some associations between these candidate genes and milk fatty acid levels and profiles have been described previously. For example, *DGAT1* has been associated with milk SFA yield (Winter *et al.*, 2002), *SCD1* associated with milk UFA ratio (Garnsworthy *et al.*, 2010), and three haplotypes (H1, H2 and H3) of *FABP4* have been revealed to affect C14:0 levels in the early stage of lactation (Nafikov *et al.*, 2013). There have been very few studies looking at the effects of genetics on milk fat production and composition in Kiwicross^{TM} cows farmed on pasture, but some potential candidate genes are described below.

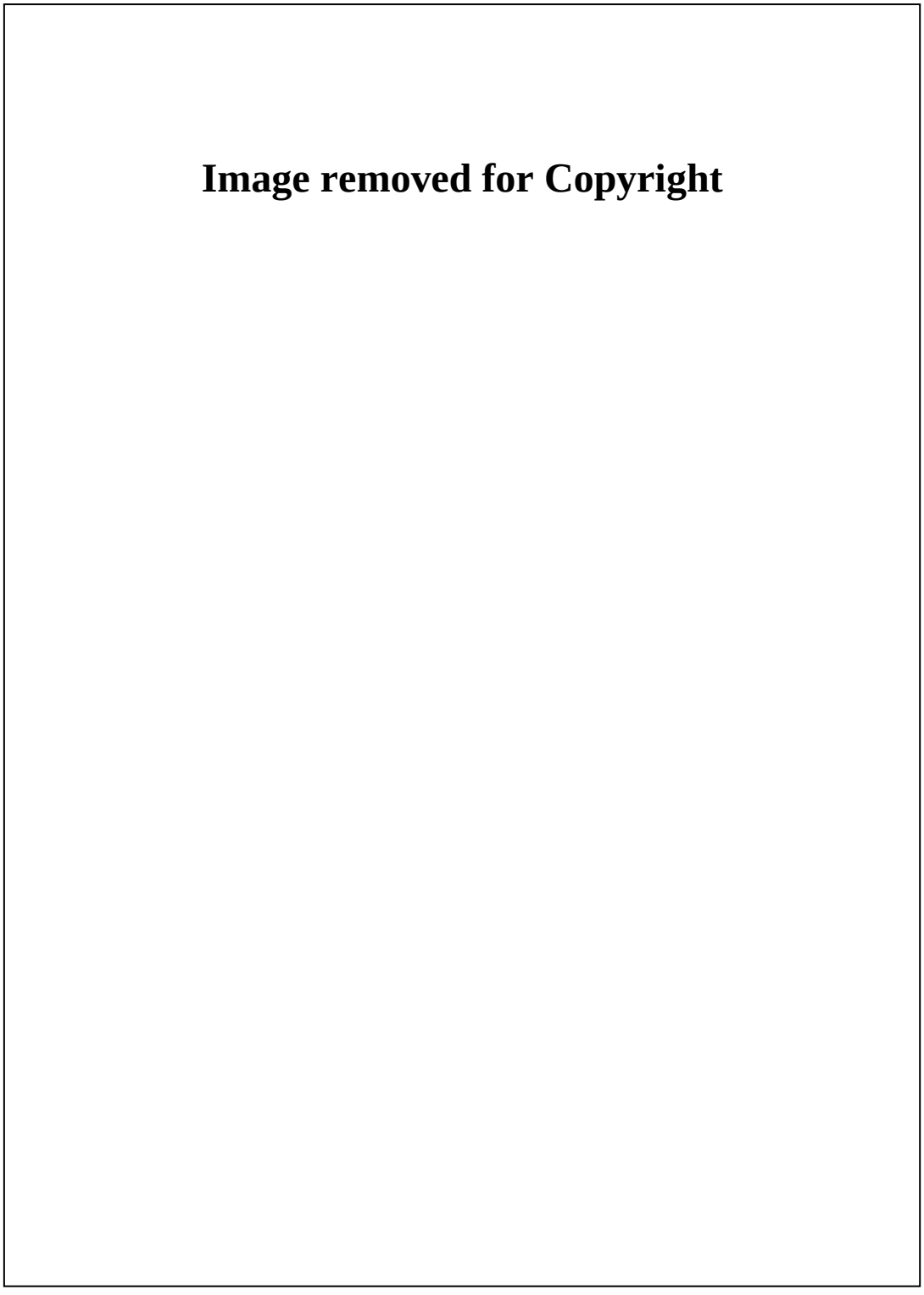


Figure 2. 8 Interrelationships among cellular pathways regulating milk fat synthesis in bovine mammary tissue (Bionaz *et al.*, 2008b).

2.3.1. The Diacylglycerol Acyl-CoA Acyltransferase 1 gene (*DGAT1*)

The gene for DGAT1 is located on bovine chromosome 14, in proximity to where a milk fat QTL was historically positioned (Coppieters *et al.*, 1998). Ogorevc *et al.* (2009) summarized the results from previous studies, and revealed the effects of many cattle candidate genes and genetic markers on milk production and mastitis in a map (Figure 2.9). Most of the studies illustrate that a location near *DGAT1* affects milk traits.

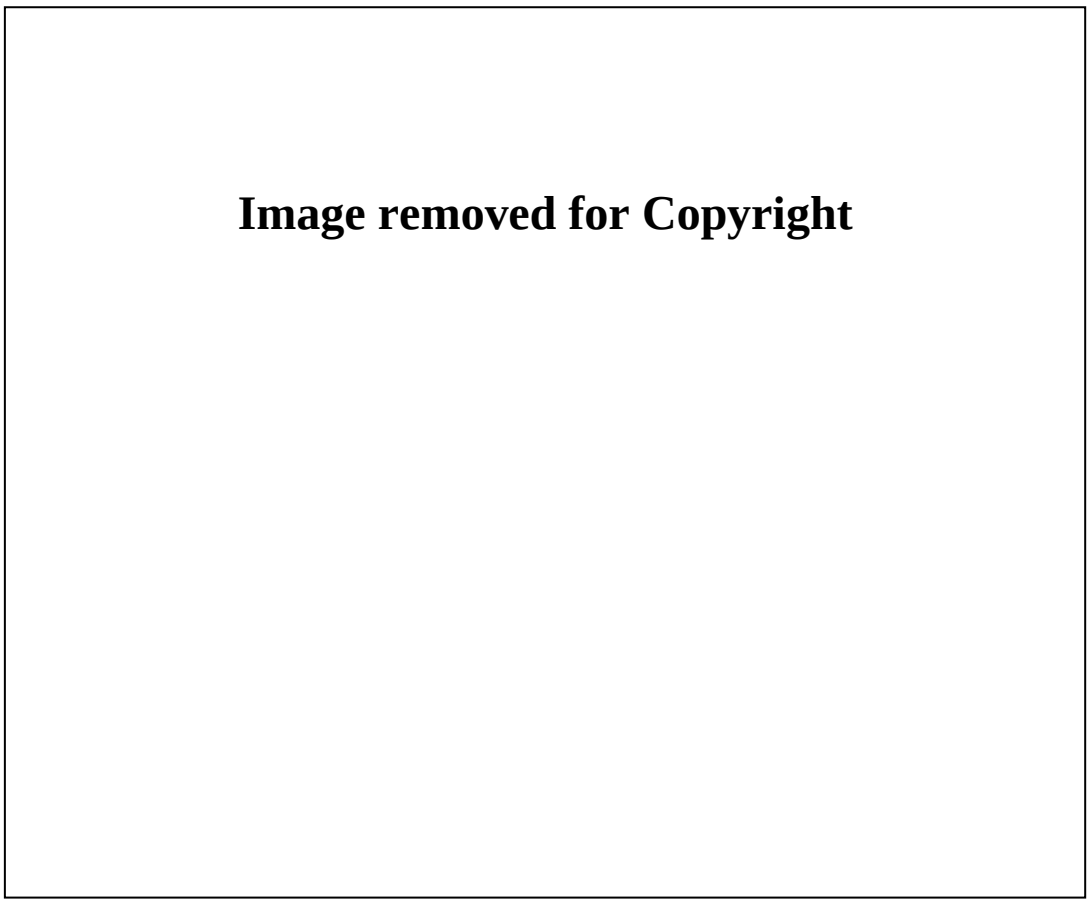


Figure 2. 9 The locations of *DGAT1* and *FABP4* on the genetic map of cattle candidate genes and genetic markers for milk production and mastitis traits. Milk yield (MY), protein yield (PY), protein percentage (PP), protein content (PC), fat percentage (FP), fat yield (FY), fat content (FC), somatic cell score (SCS), clinical mastitis (CM) (Ogorevc et al., 2009).

The protein produced by the DGAT1 gene, plays a key role in triacylglycerol (TAG) synthesis (Figure 2.8). It regulates triglyceride metabolism in the mammary gland (Chen *et al.*, 2002). In TAG synthesis, DGAT1 is the rate-limiting enzyme (Liu *et al.*, 2012) and TAG

accumulation is related directly to the expression of DGAT1 (Monetti *et al.*, 2007; Steven *et al.*, 2000). Furthermore, research suggests that milk fatty acid composition could be affected by *DGAT1* polymorphism, with an effect reported for both C16:0 and CLA (Tzompa-Sosa *et al.*, 2016).

A more detailed discussion of the activity of DGAT1 and how that may affect gross milk traits and milk FA composition occurs in a following chapter.

2.3.2. The Fatty Acid Binding Protein 4 gene (*FABP4*)

Fatty acid binding protein 4 (FABP4) is a member of the FABP family (FABP1-FABP9), a group of intra-cellular lipid-binding proteins (Zimmerman *et al.*, 1998). The main function of FABP4 is to bind long-chain fatty acids (LCFA) and transport them within animal cells, including mammary gland cells. Previous studies have demonstrated that *FABP4* plays a key role in fatty acid (FA) uptake processes in animals (Bionaz *et al.*, 2008b). In genetic map of cattle candidate genes and genetic markers for milk production and mastitis (Figure 2.9), *FABP4* is located in a region that contains QTLS for milk traits.

In the bovine lactation cycle, expression of *FABP4* was up-regulated during the first 60 days of lactation, when body lipid mobilization was occurring (Bionaz *et al.*, 2008a). Although the expression of *FABP4* declines subsequently, its expression level is still much higher than in non-lactating dairy cows. *FABP4* has therefore been proposed as a candidate gene-marker for milk production traits and milk FA composition (Khatkar *et al.*, 2004). It has also been associated with meat fat content and FA composition in beef cattle (Michal *et al.*, 2006).

2.3.3. The Stearoyl-CoA Desaturase 1 gene (*SCD1*)

Stearoyl-CoA desaturase 1 (SCD1) is an enzyme located in the endoplasmic reticulum that catalyses the rate-limiting step in the formation of monounsaturated fatty acids (MUFAs), specifically oleate (C18:1) and palmitoleate (C16:1) (Paton *et al.*, 2009). In lipid metabolism, the enzyme encoded by *SCD1* gene introduces a single double bond at the Δ9, 10 position in a range of FA (Ntambi *et al.*, 2004), especially the long-chain acyl-CoAs, and either derived from *de novo* synthesis or diet. The desaturation of a wide spectrum of monounsaturated

fatty acyl-CoA substrates can be catalysed by SCD1, such as the desaturation of C18:1 *trans*-11 to generate *cis*-9, *trans*-11 CLA (Ntambi *et al.*, 2004).

Previous studies report abundant expression of *SCD1* in the mammary gland of lactating ruminants (Bernard *et al.*, 2005; Bionaz *et al.*, 2008b; McDonald *et al.*, 1973). It is an important candidate gene associated with milk fat and FA composition (Gautier *et al.*, 2006) and is located on BTA26 (Figure 2.10).

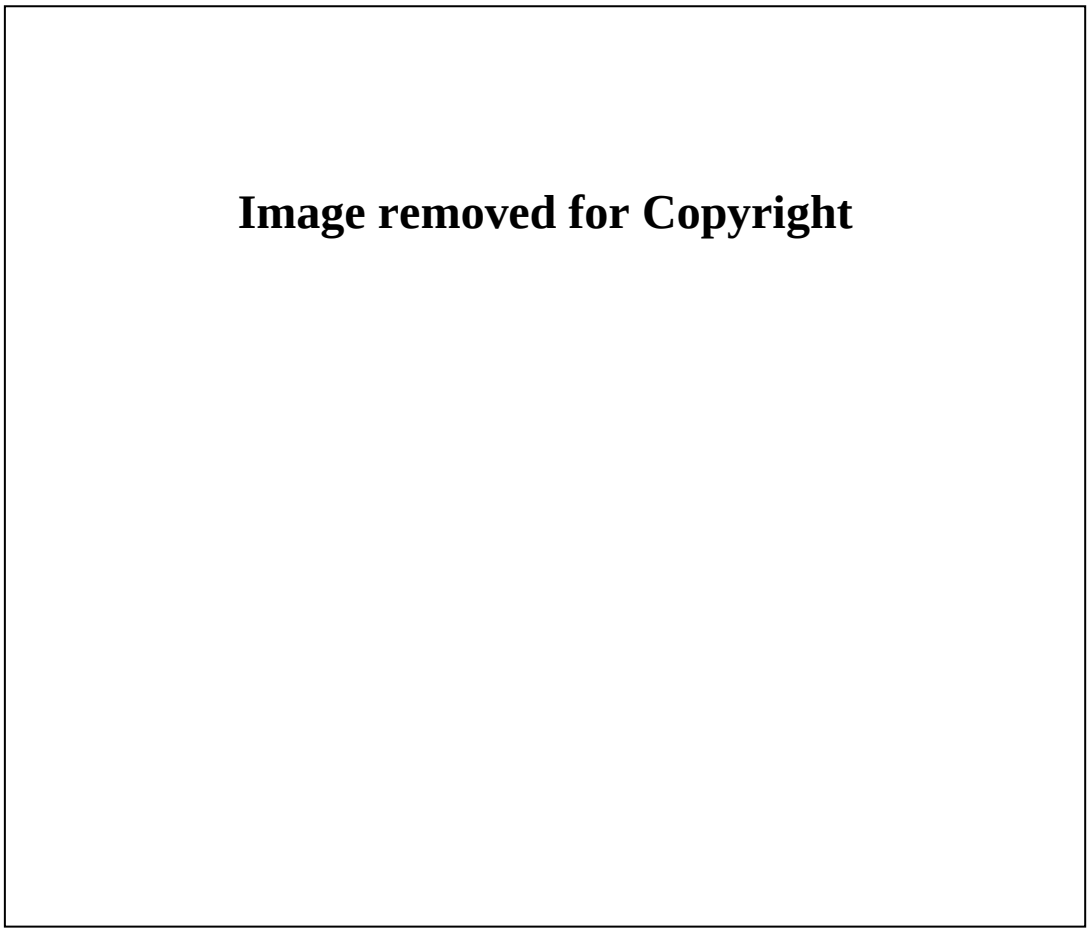


Figure 2. 10 The location of *SCD1* on the genetic map of BTA26 among QTLs for milk production and mastitis. Milk yield (MY), protein yield (PY), protein content (PC), fat percentage (FP), fat yield (FY), somatic cell score (SCS) (Ogorevc et al., 2009).

2.3.4. The Perilipin-2 gene (*PLIN2*)

The perilipin-2 gene (*PLIN2* also known as *ADFP*) encodes the protein perilipin-2 (also called the adipose differentiation-related protein (ADRP), and adipophilin). During lactation,

adipocyte differentiation-related protein participates in globule surface membrane formation, and it is one of the constituents of the globule surface (Figure 2.8). In the data base of Ogorevc *et al.* (2009), there is a study describing how *PLIN2* may be associated with the milking speed and other links between *PLIN2* (located on BTA8) and milk QTLs have been reported (Figure 2.11).

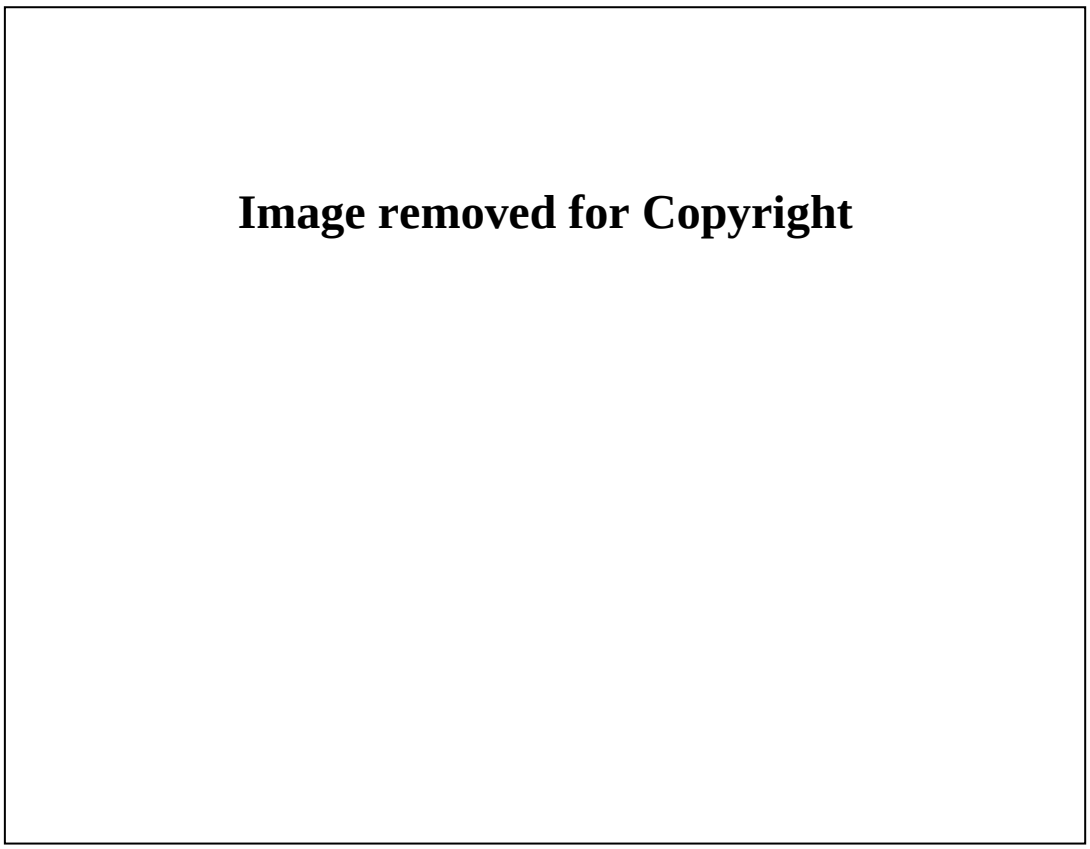


Figure 2. 11 The location of *PLIN2* on the genetic map of BTA8. Protein percentage (PP), milking speed (MSPD), somatic cell score (SCS), somatic cell count (SCC), clinical mastitis (CM) (Ogorevc *et al.*, 2009).

In other research on bovine intramuscular fat deposition and marbling, *PLIN2* is a candidate gene for fat deposition traits, because muscular tissues will uptake more fatty acid for triglyceride formation when abundant PLIN2 exists (Imai *et al.*, 2007; Imamura *et al.*, 2002; Magra *et al.*, 2006).

2.3.5. The Lipin 1 gene (*LPIN1*)

The lipin family includes three isoforms (LPIN1, 2 and 3) that are involved in dephosphorylation of phosphatidic acid to form diacylglycerol (the substrate for triglyceride). Previous studies have revealed that the lipin 1 gene (*LPIN1*) is associated with adipose tissue development and triglyceride accumulation (Phan *et al.*, 2004), and Ogorevc *et al.* (2009) reveal the gene to be located in a region on BTA11 that contains QTLs for some milk traits (Figure 2.12).

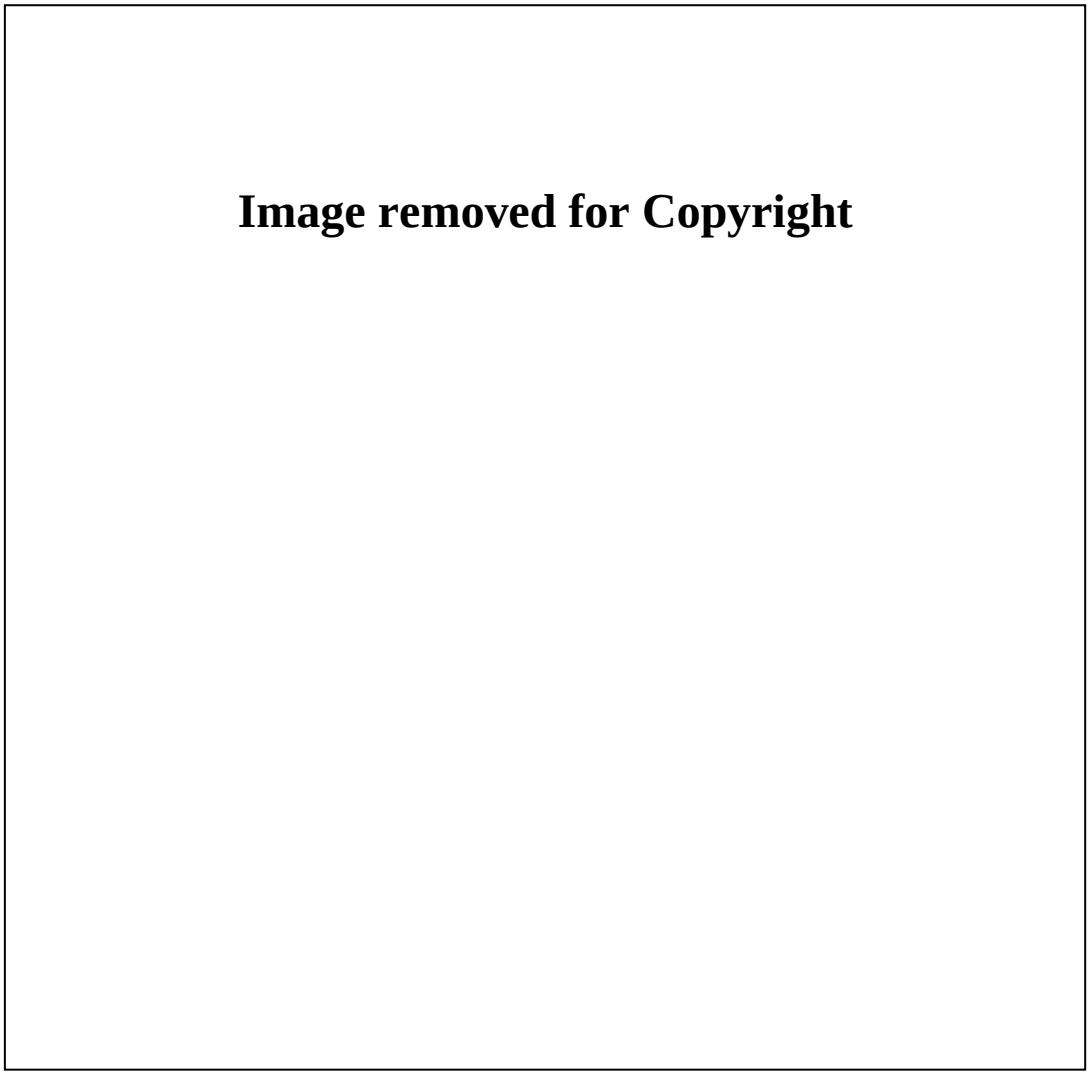


Figure 2. 12 The location of *LIPIN1* on the genetic map of BTA11. Milk yield (MY), protein percentage (PP), fat yield (FY), somatic cell score (SCS), somatic cell count (SCC), clinical mastitis (CM) (Ogorevc *et al.*, 2009).

All of the lipin isoforms are expressed in cow mammary tissues, but the mRNA of *LPIN1* was the predominant mRNA during a lactation (Bionaz *et al.*, 2008a). As the major isoform, that could affect triacylglycerol synthesis in adipose tissue and the mammary gland, genetic variation in *LPIN1* may affect milk FA synthesis.

2.4. Finding nucleotide sequence variation: the use of Polymerase Chain Reaction - Single-Stranded Conformational Polymorphism (PCR-SSCP) analysis

Genetic variation occurs across the genome of cattle. Sequence variation within or near to a gene can cause genetic disease, or be associated with variation in phenotype, because the variation may either affect the expression of the gene, or the function of the protein produced. Nucleotide sequence variation can be used to describe an animal's genetic background or pedigree. For example, it has become common to refer Single Nucleotide Polymorphisms (SNPs), single nucleotide changes in a DNA sequence (Vignal *et al.*, 2002), and describe how they can be used for disease diagnosis (Gupta *et al.*, 2001) or breed selection for production in farm animals (Fan *et al.*, 2010). While the term SNP is commonly used, the Human Genome Variation Society (HGVS), the Human Variome Project (HVP) and the HUman Genome Organisation (HUGO) recommends a nomenclature (The HGVS nomenclature – see https://varnomen.hgvs.org/) that discourages the use of the term.

To study the associations between the genetic variations and animal quantitative production traits, some researchers use DNA microarrays (also named as DNA chip or biochip) to genotype multiple regions of a genome. A DNA chip contains a lot of specific DNA sequences which can be a short section of different genes, and which can hybridize with a lot of different cDNA or cRNA targets concurrently. DNA microarrays can diagnose the well-known SNPs efficiently, but they cannot identify hitherto unidentified sequence variation. The aim of this study was therefore to try to find new variations in dairy milk production-related genes. A reliable, sensitive and accurate DNA typing technology was therefore needed.

To discover genetic variation and lay a foundation for the identification of molecular or gene markers, many different approaches can be used. These include, PCR-SSCP analysis, SNP analysis, Random Amplified Polymorphic DNA (RAPD) analysis, Amplified Fragment Length Polymorphism (AFLP) analysis and Restriction Fragment Length Polymorphism

(RFLP) analysis, and DNA sequencing. Based on the utility, simplicity and cost-effectiveness of these technologies, PCR-SSCP analysis was used in this investigation.

Orita *et al.* (1989) described how single-stranded conformational polymorphism (SSCP) was a valuable method to detect SNPs that can occur in any position in a fragment of DNA, along with various other forms of nucleotide variation including insertions and deletions. In genotyping large numbers of animals (or other species), PCR-SSCP has many advantages, including being a rapid approach, which is never-the-less accurate, cost-effective and easily interpreted. The PCR-SSCP allows for variant forms of a gene or nucleotide sequence to be identified prior to sequencing, while also enabling the isolation of single sequence templates from heterozygous samples (less numbers of clones required for plasmid DNA purification and sequencing) (Zhou *et al.*, 2008).

Conceptually PCR-SSCP resolves sequence variation in fragments of DNA that have been amplified by PCR. When denatured at higher dilution, PCR amplicons form separate single strands of DNA, which then can form stable secondary structures upon rapid cooling. These conformers can be resolved using electrophoresis (Figure 2.13).

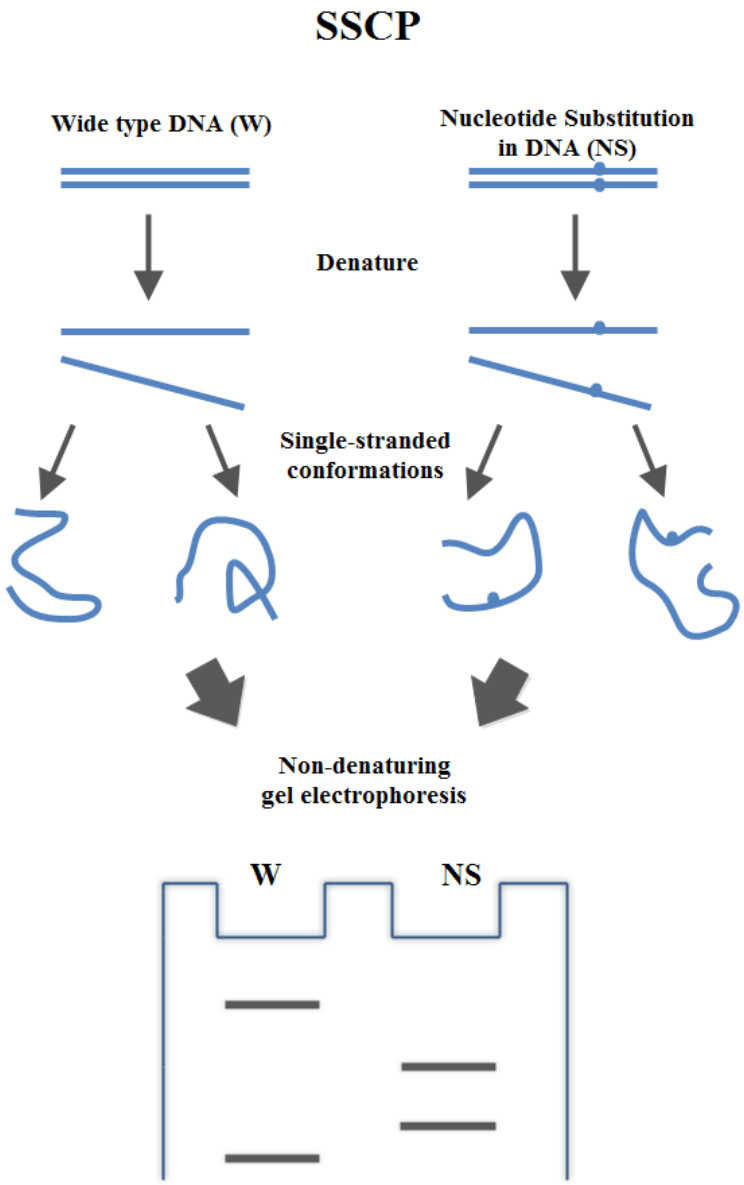


Figure 2. 13 The principle of PCR-based SSCP analysis.

Orita *et al.* (1989) and Hayashi (1999) suggested that the PCR-SSCP was better suited to investigating shorter fragments of DNA, typically fragments of less than 430bp, while Zhou *et al.* (2008) describe how the optimum size of the amplicons is between 100 and 400 bp.

Chapter 3. *DGAT1* variation and its association with variation in gross milk traits and milk fat composition

The genes that underpin key dairy traits are of ongoing interest in dairy production research. Among these genes is the diacylglycerol acyl-CoA acyltransferase 1 (DGAT1) gene (*DGAT1*). This is located on bovine chromosome 14, in proximity to where a milk fat QTL was historically positioned (Coppieters *et al.*, 1998). Sequence variation in *DGAT1* has been described, and a well-studied polymorphism results in the substitution of lysine (K) with alanine (A) at position 232 of the amino acid sequence (known as p.K232A) (Figure 3.1).

Figure 3. 1 Alignment of the amino acid sequence of DGAT1 from different mammalian species. The dash (-) represent the same amino acid in the four species; the dot (.) was a missing and the triangles (▼) was the p.K232A variations.

This was first described by Grisart *et al.* (2002), where the variation was associated with various milk traits. Subsequently, analysis of *DGAT1* variation in NZ dairy cows (Spelman *et al.*, 2002) suggested that the average allele substitution effects were 2-3 kg of protein and 120-130 L of milk for both the Jersey and Holstein Friesian breeds, with a substitution effect of 6 kg of milk fat for Holstein-Friesians and 3 kg for Jersey cows. The effect of p.K232A in the Kiwicross™ cow could not be assessed as these genetics was not released by LIC until 2005.

In 2007, Schennink *et al.* (2007) reported how the p.K232A variation in *DGAT1* affected milk traits and milk fatty acid (FA) composition. They found that the K allele was associated with a decreased milk yield and increased milk fat content. In addition, the K allele was also associated with a higher concentration of SFA and C16:0 FA, but a lower concentration of C14:0 FA, unsaturated C18 FA and Conjugated Linoleic Acid (CLA). These results suggest the effects of p.K232A, would make it a useful gene-marker for the selective breeding of dairy cattle.

While the association between p.K232A and milk fat traits had been investigated in pasture-fed NZ Holstein-Friesian and Jersey cows (Spelman *et al.*, 2002), few studies describe the effect of p.K232A on milk fat composition in a pasture-based system, and specifically in the now dominant NZ Kiwicross™ cow. This laid the basis for the following investigation.

Outside of exon 8 of *DGAT1* (where the nucleotide sequence variation creating p.K232A is located), Klaus *et al.* (2015) found further variation in bovine *DGAT1* at position c.1303A>C in exon 16. This non-synonymous substitution would cause the amino acid at the position 435 to change from Met to Leu, and would lead to the DGAT1 protein being unable to transfer oleic acid from CoA to diacylglycerol. The cows with this mutation produce milk with less SFA, but more MUFA. This finding suggests a novel way that one could change the nutritional properties of milk, using marker-assisted selection.

Alignment of the amino acid sequence for *DGAT1* from different species reveals that the lysine residue at position 232 is conserved across species (Figure 3.1). Equally, variation at the leucine residue at position 435 reported by Klaus *et al.* (2015) appears to be unique to the bovine sequence.

It had been suggested that the p.K232A polymorphism cannot explain the strength of the association between *DGAT1* and milk traits in all breeds (Rosse Ida *et al.*, 2014). One reason for this may be that variation elsewhere in the gene is actually causing the variation in milk

fat, and not p.K232A specifically. For example, variations in the 3'UTR of *DGAT1* have been identified to be candidate gene markers, such as the insertion-deletion (INDEL) polymorphism (DNA sequence: TGGTCAGACGTCTTG; position: c.1467+412 to c.1467+426) in *Bos indicus* cattle. Furthermore, a variable number of tandem repeat (VNTR) polymorphism in the promoter region of cattle *DGAT1* has been reported to have effects on milk fat content (Kuhn *et al.*, 2004). Research has been undertaken on *DGAT1* in buffaloes (*Bubalus bubalis*) and how the same VNTR polymorphism in that species influences dairy buffalo milk fat percentage (Cardoso *et al.*, 2015).

To investigate the effects of *DGAT1* variation on milk traits in Kiwicross™ cows, different regions of this gene were chosen for investigation. These regions included parts of the promoter, exons, introns and downstream regions. These regions were chosen so as to contain the reported variations, including p.K232A, the VNTR and the INDEL.

3.1. Materials and Methods

3.1.1. Animals and milk sample collection

This research was approved by the Lincoln University Animal Ethics Committee (AEC Number 521) under the provisions of the Animal Welfare Act 1999 (NZ Government).

A total of 395 Kiwicross™ cows, of variable and unknown Holstein-Friesian and Jersey breed proportion, and of 3-10 years of age were studied. These were obtained from two herds: 113 cows in herd 1, and 282 cows in herd 2. All the cows were grazed on pasture (a mixture of perennial ryegrass and white clover) on the Lincoln University Dairy Farm (LUDF; Canterbury, NZ). All the cows calved over the months August-September, and they were milked twice a day.

Blood sample from each cow was collected onto an FTA card and allowed to air dry. Genomic DNA was purified from a 1.2 mm punch of the dried blood spot using a two-step washing procedure as described by Zhou et al. (2006).

Samples for milk trait analyses were collected once a month from September 2013 to February 2014. The daily milk yield in litres was recorded using Tru-test milk meters (Tru-test Ltd, Auckland, NZ). These samples were analysed for fat percentage (%) and protein percentage (%) using Fourier-Transform Infra-Red Spectroscopy (MilkoScan FT 120 Foss,

Hillerød, Denmark). The milk samples for FA analysis were collected from each cow in a single afternoon milking on 15th January 2014 (days in milk (DIM) = 148 ± 19 days). These were frozen at -20 ºC, and then freeze-dried, prior to being individually ground to a fine powder for component analysis.

3.1.2. Gas Chromatography of the Fatty Acids in the Milk Sample

The milk FAs were methylated and extracted in n-heptane, before being analysed by Gas Chromatography (GC) as FA methyl esters (FAMEs). The methylation reactions for ester formation were performed in 10-mL Kimax tubes. Individual powdered milk samples (0.17 g), were dissolved in 900 µL of n-heptane (100%, AR grade), before 100 µL of internal standard (5 mg/ml of C21:0 methyl ester in n-heptane) and 4.0 mL of 0.5 M NaOH (in 100% anhydrous methanol) were added. The tubes were vortexed then incubated in a block heater (Ratek Instruments, Australia) at 50 ºC for 15 minutes. After cooling to room temperature, another 2.0 mL of n-heptane and 2.0 mL of deionized water was added to each tube. After vortexing, the tubes were centrifuged for 5 minutes at 1500 *g* (Megafuge 1.0R, Heraeus, Germany). The top layer of n-heptane was transferred with a Pasteur pipette into a second Kimax tube, and another 2.0 mL of n-heptane was added to each of the original tubes. The extraction was repeated and the n-heptane aspirates were then pooled. Finally, anhydrous sodium sulphate (10 mg) was added to the n-heptane extracts, to remove any residual water.

The GC analysis was carried out using a Shimadzu GC-2010 Gas Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionization detector and an AOC-20i auto sampler. The output was analysed with GC Solution Software (Shimadzu). For analysis, 1 µL of the n-heptane sample extract was injected into a 100 metre GC column (250 µm × 0.25 µm capillary column, CP-Select, Varian) with a 1:60 split ratio. The separation was undertaken with a pure helium carrier gas and was run for 92 minutes. The temperature of both the injector and detector were set at 250 ºC and the thermal profile of the column consisted of 45 ºC for 4 minutes, followed by 27 minutes at 175 ºC (ramped at 13 ºC /minute), 35 minutes at 215 ºC (ramped at 4 ºC /minute.), and a final 'bake-off' at 250 ºC for 5 minutes (ramped at 25 ºC /minute.). The individual FAMEs were identified by the peak retention time compared to commercially obtained external standards (ME61, ME93, BR3, BR2, ME100, GLC411 and GLC463; Laroden AB, Sweden). Quantification of the individual FAMEs was based on peak area assessment and comparison with the internal and external standards. The threshold for

peak area determination on the chromatogram was a 500-unit count, with peaks that were under 500-unit count, being ignored. The calculated minimum component of an individual FAME was therefore 0.01 g per 100 g of total FA.

The individual FA measurement and grouped FA measurement are listed in Table 3.3 and 3.4, and the mean levels in the 395 cows calculated. These groups were, short chain FAs (SCFA) = C4:0 + C6:0 + C8:0; medium chain FAs (MCFA) = C10:0 + C12:0 + C14:0; long chain FAs (LCFA) = C15:0 + C16:0 + C17:0 + C18:0 + C19:0 + C20:0 + C22:0 + C24:0; omega 3 FAs = C18:3 *cis*-9, 12, 15 + C20:5 *cis*-5,8, 11, 14, 17 + C22:5 *cis*-7, 10, 13, 16, 19; omega 6 FAs = C18:2 *cis*-9, 12 + C18:3 *cis*-6, 9, 12 + C20:3 *cis*-8, 11, 14 + C20:4 *cis*-5, 8, 11, 14; monounsaturated FAs (MUFA) = C10:1 + C12:1 + C14:1 *cis*-9 + C15:1 + C16:1 *cis*-9 + C17:1 + C18:1 *trans*-11 + C18:1 *cis*-9 + C18:1 *cis*-(10 to 15) + C20:1 *cis*-5 + C20:1 *cis*-9 + C20:1 *cis*-11 + C22:1 *trans*-13; polyunsaturated FAs (PUFA) = C18:2 *trans*-9, 12 + C18:2 *cis*-9,trans-13 + C18:2 *cis*-9,*trans*-12 + C18:2 *trans*-9,*cis*-12 + C18:2 *cis*-9, 12 + C18:3 *cis*-6, 9, 12 + C18:3 *cis*-9, 12, 15 + CLA + C20:3 *cis*-8, 11, 14 + C20:4 *cis*-5, 8, 11, 14 + C20:5 *cis*-5, 8, 11, 14, 17 + C22:5 *cis*-7, 10, 13, 16, 19; total branched FA= C13:0 *iso* + C13:0 *anteiso* + C15:0 *iso* + C15:0 *anteiso* + C17:0 *iso*; total UFA = MUFA + PUFA; and total SFA = C4:0 + C6:0 + C8:0 + C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C19:0 + C20:0 + C22:0 + C24:0.

Unsaturated FA ratios and indices were also calculated as follows: total index (total UFA divided by the sum of total SFA and total UFA); MUFA index (MUFA divided by the sum of MUFA, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0 and C22:0); C10:1 index (C10:1 divided by the sum of C10:0 and C10:1); C12:1 index (C12:1 divided by the sum of C12:0 and C12:1); C14:1 index (C14:1 *cis*-9 divided by the sum of C14:0 and C14:1 *cis*-9); C16:1 index (C16:1 *cis*-9 divided by the sum of C16:0 and C16:1 *cis*-9); C18:1 index (C18:1 *cis*-9 divided by the sum of C18:0 and C18:1 *cis*-9); and CLA index (CLA divided by the sum of CLA and C18:1 *trans*-11).

3.1.3. PCR primers used for dairy cattle *DGAT1* amplification

Blood samples were analysed at the Lincoln University Gene-Marker Laboratory. Eight sets of PCR primers were designed using the reference sequence AYO65621.1 and these are listed in Table 3.1, with the regions of the gene amplified illustrated in Figure 3.2.

Table 3. 1 Primers used to amplify eight regions of the dairy cattle *DGAT1*.

DGAT1 region[1]	Amplicon size (bp)	Forward primer	Reverse primer
1	364	5'-TTCGCTGTGACCCTGGCAG-3'	5'-TCCAGTCTCCTTTGCCTTCG-3'
2	154	5'-TGCATTTGCCAGGAGACCAC-3'	5'-CGCCTCTACTACGCCACTG-3'
3	393	5'-CTCAACTTCTAGACGCCCTC-3'	5'-CATCAGTCCTTCAGCTAAGC-3'
4	349	5'-CACAGGTGAGTGGTCTTGG-3'	5'-CAGACACGTCATCTGGAGG-3'
5	374	5'-CCACTGGGCTGCCACTTG-3'	5'-GAAGCAAGCGGACAGTGAG-3'
6	410	5'-ACCAGGCACCGGGGCTCAG-3'	5'-GCAGAGTGGGCAGGGGCTC-3'
7	493	5'-TGGTGGTGGGTGGCCTTGC-3'	5'-GAGTGAGCCTCTGCGCTCG-3'
8	252	5'-CTGTGCACAGTGAGCTCC-3'	5'-GTTCAAGTCAGCCTGATTCTC-3'

[1] **See Figure 3.2**

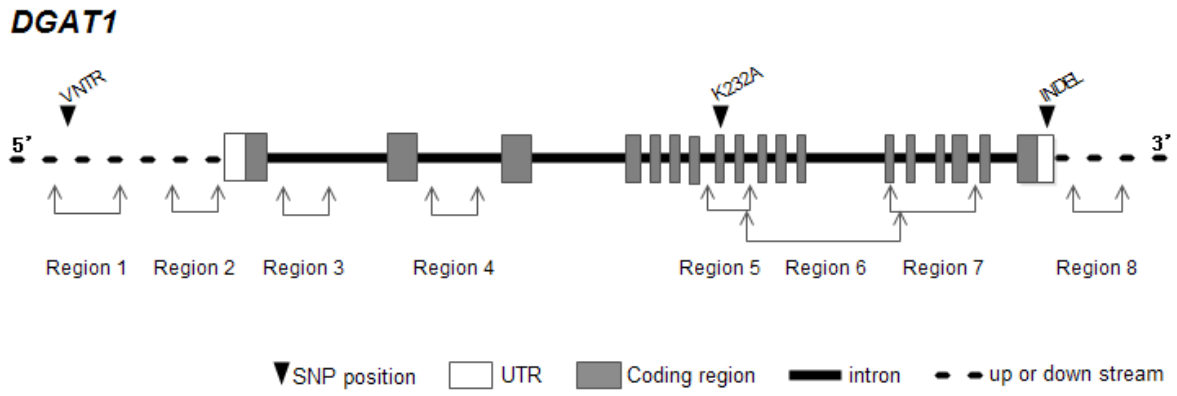


Figure 3. 2 Location of *DGAT1* regions that were amplified. Eight sets of primers were designed in order to amplify a 364 bp and 154 bp region in the promoter; a 393 bp region in intron 1; a 349 bp region in intron 2; a 374 bp region spanning intron 7 - intron 9, a 410 bp region spanning intron 9 - intron 13; a 493 bp region spanning exon 13 - intron 16, and a 252 bp region in the 3'UTR. The gene structure is based on the cattle *DGAT1* sequence, and is not drawn to scale. Cattle *DGAT1* has 18 exons. (Reference sequence number: AYO65621.1).

3.1.4. Developing the PCR-SSCP protocol for *DGAT1*

Cattle DNA samples (n = 25) were used to develop the PCR-SSCP protocols for analysis of the target regions of *DGAT1*. The PCR protocols were optimised with different annealing temperatures (between 50 ^{o}C and 62 ^{o}C). Electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels was used to visualise the target amplicons, with 1× TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide.

When the agarose gels produced a satisfactory result, the conditions for band separation and resolution were optimised at different percentage polyacrylamide gels (10 %, 12 % and 14 %), different concentrations of glycerol (0.5 % - 4.0 %) and at various temperatures (3 ^{o}C – 35 ^{o}C). For the first test, amplicons were loaded onto 16 cm × 18 cm, 14% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 250 V for 19 hours at 15 ^{o}C in 0.5× TBE buffer.

3.1.5. PCR-SSCP analysis and genotyping of dairy cattle *DGAT1*

A blood sample from each of the experimental cows was collected onto FTA cards and air dried. Genomic DNA was purified from a 1.2-mm punch of the dried blood spot, using a two-step washing procedure, as described by Zhou *et al.* (2006).

After optimising the PCR amplification protocol for each target region, amplification of Region 2, 3, 4 and 6 were performed in a 15-µL reaction containing the genomic DNA (punch of FTA paper), 0.25 µM of each designed primer, 150 µM of each dNTP (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the polymerase enzyme. For Region 5, the PCR amplification was performed in a 15-µL reaction containing the genomic DNA on the FTA punch, 0.25 µM of each designed primer, 150 µM of each dNTP, 0.95 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase and 1× the reaction buffer supplied with the polymerase enzyme. For Region 1 and 8, the PCR amplifications didn't work.

Amplification was undertaken using S1000 thermal cyclers (Bio-Red, Hercules, CA, USA) and the thermal profile included an initial denaturation for 2 minutes at 94 ^{o}C; followed by 35 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 58 ^{o}C and 30 seconds at 72 ^{o}C; with a final extension for 5 minutes at 72 ^{o}C. Following amplification, a 0.7-µL aliquot of the PCR products was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ^{o}C for 5 minutes and rapid cooling on wet ice, the samples were loaded on 16 cm × 18 cm, acrylamide: bisacrylamide (37.5: 1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) in 0.5× TBE buffer. The method of Byun *et al.* (2009) was used to silver-stain the gels.

For Region 5, amplicons were loaded onto 16 cm × 18 cm, 14% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 280 V for 19 hours at 26 ^{o}C in 0.5× TBE buffer.

For Region 2, 3, 4 and 6, amplicons were loaded onto 16 cm × 18 cm acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels with different percentages and electrophoresis was performed using Protean II xi cells (Bio-Rad), at different voltages and temperatures in 0.5× TBE buffer.

3.1.6. Sequencing of the dairy cattle *DGAT1* Region 5 variants

Homozygous PCR amplicons identified by PCR-SSCP were sequenced at the Lincoln University DNA Sequencing Facility. If there were not any homozygous samples, single bands of interest from the heterozygous were recovered directly from the SSCP gels as a gel slice. This was macerated and the DNA was eluted into 50 µL TE buffer by incubating at 70 ^{o}C for 20 minutes. The original primers and 1 µL of the eluted solution (as a template) were used for a second round of PCR amplification to produce a simple SSCP gel pattern which could be directly compared to, or found in, the pattern derived from the original heterogeneous amplicons. When banding patterns could be matched and identified, then the second PCR amplicons were directly sequenced at the Lincoln University DNA Sequencing Facility. The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment, translations and comparisons. The BLAST algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

3.1.7. Statistical analysis

Hardy-Weinberg equilibrium (HWE) for the *DGAT1* genotypes was analysed using an online chi-square calculator (http://www.oege.org/software/hwe-mr-calc.shtml).

All other statistical analysis was carried out using IBM SPSS version 22 (IBM, NY, USA), with associations between the genotypes that result in p.K232A and variation in milk traits and milk FA component levels being tested using General Linear Mixed-effects Models (GLMMs). First, a GLMM (fixed effect: genotype, days in milk, age and herd) and multiple

pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of the different genotypes on milk production traits. Days in milk (DIM) was counted from the day of calving. Next, a GLMM (fixed effect: genotype, DIM, age and herd) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes on milk FA component levels.

The effect of sire of cow could not be included in the GLMMs. Some semen straws (sire genetics) used in NZ dairy cattle artificial insemination-based breeding approaches, contain mixed-sire semen purchased from commercial semen producers. In these cases, individual sire identity is impossible to ascertain, but because the straws were mixed-semen straws and because different sires are used for different inseminations, in different years, it is unlikely that sire was a strongly confounding effect. Cow age and herd might also therefore be confounded with sire, but this cannot be confirmed.

3.2. Results

3.2.1. Sequence variation in *DGAT1*

Amplicons were not obtained with the primers for Regions 1 and 8. The sequences obtained for Regions 2, 3, 4, 6 and 7, didn't show any nucleotide variations in the 395 cows analysed. In Region 5, the nucleotide variation that results in p.K232A was observed in exon 8 (Figure 3.3), but the intron 8 and exon 9 sequences didn't have any detectable nucleotide sequence variation. In the cattle analysed, *DGAT1* genotypes that would produce AA, AK, and KK at p.K232A were found (Figure 3.4), and with frequencies of 14.9%, 46.3% and 38.8% respectively. The most frequent variant was K (61.9%) and the frequency of A was 38.1%. The *P*-value for the chi-square for deviation from HWE was 0.724, suggesting the population was at equilibrium.

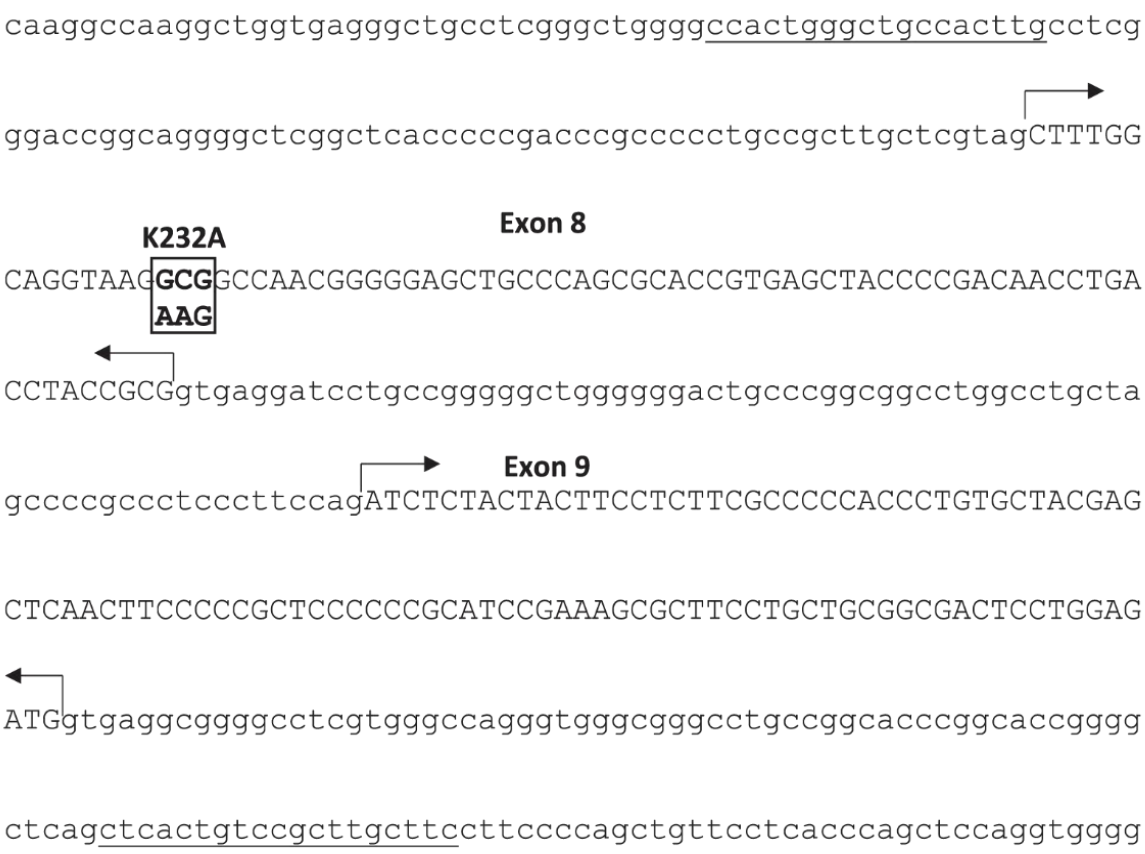


Figure 3. 3 Location of the diacylglycerol acyl-CoA acyltransferase 1 gene (*DGAT1*) primers. Nucleotides in exons are shown in upper case while those in introns are shown in lower case, with exon/intron boundaries marked with arrows. The primer binding regions are underlined, and the p.K232A codon is indicated.

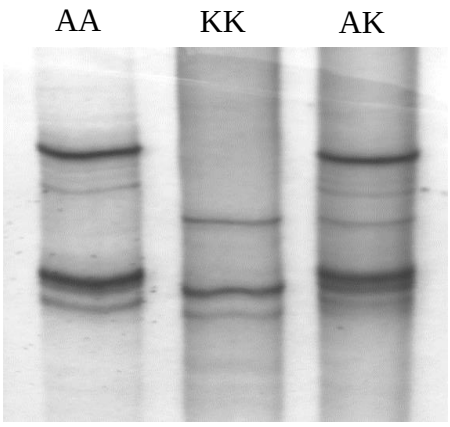


Figure 3. 4 PCR-SSCP patterns of the p.K232A variant of *DGAT1*.

3.2.2. Milk production traits and variant p.K232A

For the GLMMs assessing the effect of *DGAT1* p.K232A on gross milk traits, associations were found between the three genotypes and average daily milk yield, and fat and protein percentage levels (Table 3.2). Genotype AA was associated with a reduced content of milk fat and protein compared to the cows of genotype AK and KK. Genotype AA cows had a higher ($P < 0.001$) milk production (25.132 ± 0.609 L/day), than AK cows (23.923 ± 0.525 L/day) and KK cows (22.441 ± 0.526 L/day).

Table 3. 2 Associations between the *DGAT1* p.K232A genotypes and gross milk production traits

	Mean ± SE[1]			
	AA	**AK**	**KK**	***P***
	n = 59	n = 183	n = 153	
Average milk volume (L)	25.132 ± 0.609[a]	23.923 ± 0.525[b]	22.441 ± 0.526[c]	**<0.001**
Fat percentage (%)	4.331 ± 0.077[c]	4.840 ± 0.066[b]	5.271 ± 0.067[a]	**<0.001**
Protein percentage (%)	3.823 ± 0.049[b]	3.919 ± 0.042[b]	4.073 ± 0.043[a]	**<0.001**

[1] Predicted means and standard error of those means were derived from the GLMMs. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted as fixed effects. Values within a row with different superscripts differ at $P < 0.05$.

3.2.3. The Fatty Acid Composition of Milk

Forty-six FAMEs were detected at levels over the threshold value and a sample output from the GC is shown in Figure 3.5. Table 3.3 summarises the average FA composition of the milk samples analysed in this investigation. The least abundant FAME (given the detection threshold) was C20:3 *cis*-8, 11, 14 FA (0.030 ± 0.000%), and the most abundant was C16:0 FA (37.623 ± 0.160%). The average abundance of the grouped FA and indices are summarised in Table 3.4.

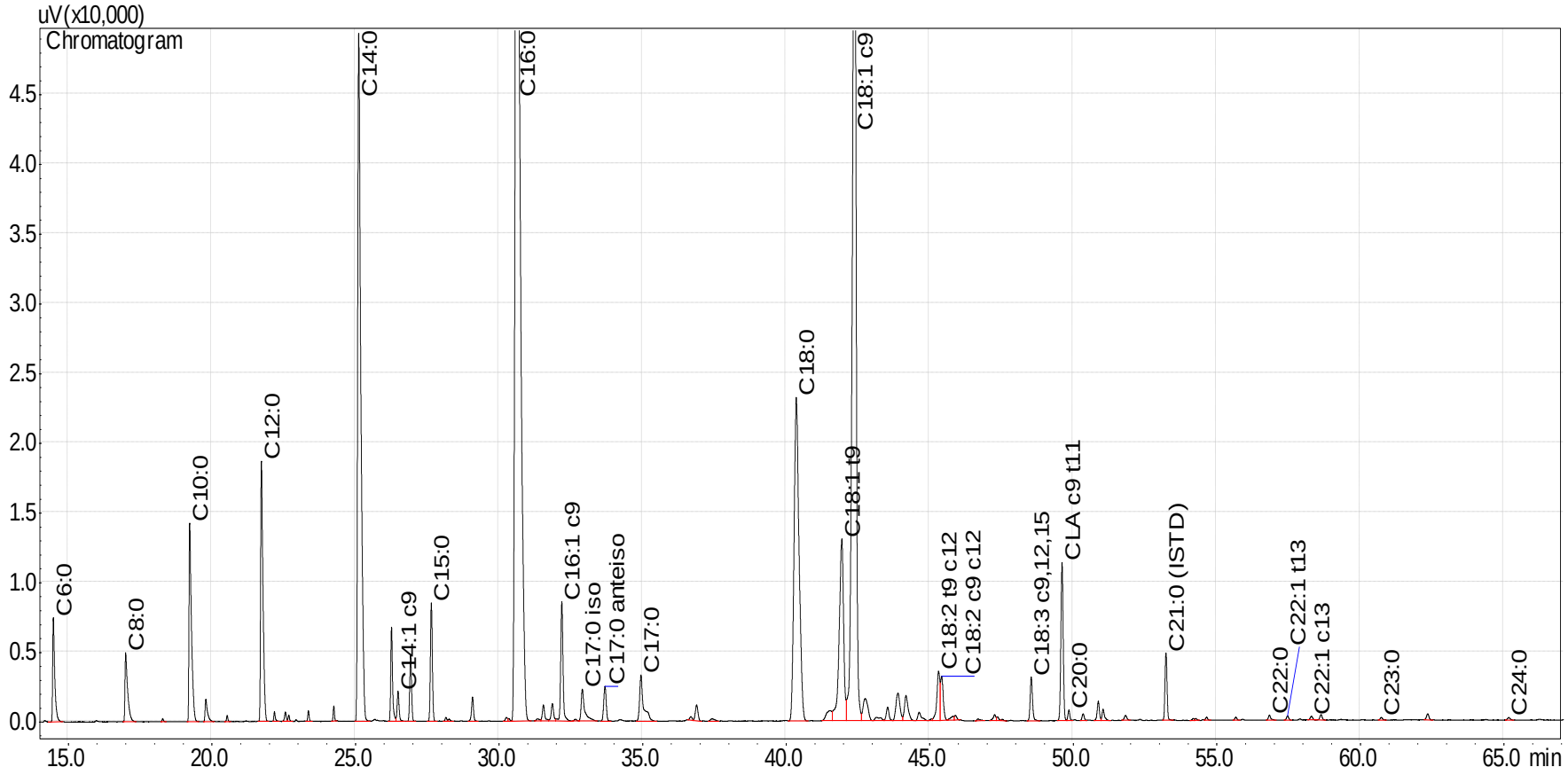


Figure 3. 5 Sample GC output of milk fatty acid methyl ester (FAME) profile from late-lactation New Zealand (NZ) HF × J-cross (KiwicrossTM) cows grazing on pasture. The individual FAME profile was identified by the peak retention time compared with external standards. Quantification of the peak areas was based on comparison with the internal standard (ISTD) C21:0.

Table 3. 3 Average quantity of individual milk fatty acid methyl ester (FAME) (means ± SE) in late-lactation, mixed-age New Zealand (NZ) HF × J-cross (Kiwicross^{TM}) cows grazing on pasture.

FAME (n = 455)	g/100 g total FAs	FAME (n = 455)	g/100 g total FAs
C4:0	1.273 ± 0.006	C18:1 *trans*-11	2.677 ± 0.041
C6:0	1.564 ± 0.005	C18:1 *cis*-9	13.052 ± 0.076
C8:0	1.183 ± 0.004	C18:1 *cis* (10 to 12)	0.505 ± 0.003
C10:0	3.231 ± 0.017	C18:2 *trans*-9,12	0.415 ± 0.002
C10:1	0.281 ± 0.002	C18:2 *cis*-9, *trans*-13	0.287 ± 0.002
C11:0	0.057 ± 0.001	C18:2 *cis*-9, *trans*-12	0.072 ± 0.001
C12:0	3.909 ± 0.024	C18:2 *trans*-9, *cis*-12	0.468 ± 0.006
C13:0 *iso*	0.078 ± 0.001	C18:2 *cis*-9,12	0.703 ± 0.004
C12:1	0.089 ± 0.001	C19:0	0.143 ± 0.001
C13:0 *anteiso*	0.037 ± 0.000	C18:3 *cis*-6,9,12	0.076 ± 0.001
C13:0	0.118 ± 0.001	C18:3 *cis*-9,12,15	0.817 ± 0.005
C14:0	12.447 ± 0.042	CLA[1]	0.968 ± 0.016
C14:1 *cis*-9	0.946 ± 0.010	C20:0	0.132 ± 0.001
C15:0 *iso*	0.292 ± 0.001	C20:1 *cis*-5	0.059 ± 0.001
C15:0 *anteiso*	0.635 ± 0.005	C20:1 *cis*-9	0.151 ± 0.001
C15:0	1.492 ± 0.008	C20:1 *cis*-11	0.076 ± 0.001
C15:1	0.284 ± 0.002	C20:3 *cis*-8,11,14	0.030 ± 0.000
C16:0	37.623 ± 0.160	C20:4 *cis*-5,8,11,14	0.035 ± 0.000
C16:1 *cis*-9	1.265 ± 0.012	C22:0	0.065 ± 0.001
C17:0 *iso*	0.563 ± 0.003	C22:1 *trans*-13	0.066 ± 0.001
C17:0	0.889 ± 0.004	C20:5 *cis*-5,8,11,14,17	0.088 ± 0.001
C17:1	0.200 ± 0.001	C24:0	0.045 ± 0.000
C18:0	8.650 ± 0.061	C22:5 *cis*-7,10,13,16,19	0.125 ± 0.001

[1] CLA = conjugated linoleic acid 'C18:2 *cis*-9, *trans*-11'.

Table 3. 4 Average quantity of grouped milk fatty acid methyl esters (FAMEs) (means ± SE) and various FA indexes in mixed age New Zealand (NZ) HF × J-cross (Kiwicross^{TM}) cows grazing on pasture.

Grouped FAME (n = 455)	g/100 g Total FAs	Desaturation index	%
SCFA	4.020 ± 0.013	Total index	25.750 ± 0.130
MCFA	19.588 ± 0.076	C10:1 index	8.062 ± 0.067
LCFA	49.039 ± 0.139	C12:1 index	2.224 ± 0.017
Total C18:1	16.524 ± 0.097	C14:1 index	7.072 ± 0.073
Total C18:2	2.915 ± 0.024	C16:1 index	3.253 ± 0.027
Total C18:3	0.893 ± 0.005	C18:1 index	65.647 ± 0.162
Omega 3	1.044 ± 0.006	CLA index	26.419 ± 0.136
Omega 6	0.845 ± 0.004		
MUFA	19.79 ± 0.099		
PUFA	4.101 ± 0.025		
Total branched FA	1.605 ± 0.007		
Total UFA	23.891 ± 0.118		
Total SFA	68.912 ± 0.131		

3.2.4. Milk fatty acid components and p.K232A variation

Table 3.5 summarises the associations revealed between *DGAT1* p.K232A variation, and 15 of the individual FAs and 15 of the grouped FAs. Of the FAs detected in this study, results were not presented if an association was not found in any of the variants.

Compared with variant K, variant A was associated with lower total saturated FA content and higher total unsaturated FA content. Variant A was also associated with lower levels of C6:0, C11:0, C13:0, C16:0, and C16:1 FA, and total LCFA, but increased C13:0 *anteiso* C14:0, C15:0 *iso*, C15:0 *anteiso*, C15:1, C17:1, C18:1 c9 FA, and CLA, MCFA, Total C18:1, Total C18:2, Total C18:3, Omega 3, Omega 6, MUFA, PUFA, branched FA, Total UFA, Total index, C18 index and CLA index.

Table 3. 5 Association between milk fatty acid levels and *DGAT1* p.K232A genotypes

FAME	Mean FAME level ± SE[2] (g/100g milk FA)			*P*
	AA	**AK**	**KK**	
	n = 59	n = 183	n = 153	
C6:0	1.534 ± 0.020[b]	1.564 ± 0.018[ab]	1.587 ± 0.018[a]	**0.007**
C8:0	1.159 ± 0.017[b]	1.192 ± 0.015[ab]	1.194 ± 0.015[a]	**0.037**
C11:0	0.049 ± 0.003[c]	0.056 ± 0.003[b]	0.063 ± 0.003[a]	**< 0.001**
C13:0 *anteiso*	0.039 ± 0.001[a]	0.037 ± 0.001[a]	0.035 ± 0.001[b]	**< 0.001**
C13:0	0.109 ± 0.005[b]	0.115 ± 0.004[ab]	0.122 ± 0.004[a]	**0.002**
C14:0	12.977 ± 0.150[a]	12.657 ± 0.129[b]	12.176 ± 0.130[c]	**< 0.001**
C15:0 *iso*	0.294 ± 0.005[a]	0.290 ± 0.004[a]	0.280 ± 0.004[b]	**< 0.001**
C15:0 *anteiso*	0.657 ± 0.017[a]	0.635 ± 0.015[a]	0.607 ± 0.015[b]	**0.001**
C15:1	0.286 ± 0.006[a]	0.280 ± 0.005[a]	0.270 ± 0.005[b]	**0.001**
C16:0	35.739 ± 0.534[c]	37.018 ± 0.460[b]	38.437 ± 0.461[a]	**< 0.001**
C16:1	1.267 ± 0.046[b]	1.352 ± 0.040[ab]	1.415 ± 0.040[a]	**< 0.001**
C18:1 *cis*-9	14.399 ± 0.275[a]	13.378 ± 0.237[b]	12.825 ± 0.237[c]	**< 0.001**
C18.2 *cis*-9, *trans*-13	0.308 ± 0.006[a]	0.284 ± 0.005[b]	0.267 ± 0.005[c]	**< 0.001**
C18.2 *cis*-9, *trans*-12	0.076 ± 0.003[a]	0.071 ± 0.003[ab]	0.066 ± 0.003[b]	**0.001**
C18.2 *cis*-9, 12	0.748 ± 0.015[a]	0.700 ± 0.013[b]	0.672 ± 0.013[c]	**< 0.001**
C18.3 *cis*-6, 9, 12	0.080 ± 0.002[a]	0.074 ± 0.002[b]	0.070 ± 0.002[c]	**< 0.001**
C18.3 *cis*-9, 12, 15	0.830 ± 0.021[a]	0.783 ± 0.018[b]	0.751 ± 0.018[c]	**< 0.001**
CLA	1.070 ± 0.054[a]	0.951 ± 0.046[b]	0.864 ± 0.046[c]	**< 0.001**
MCFA	20.069 ± 0.287[a]	19.904 ± 0.247[a]	19.343 ± 0.248[b]	**0.001**
LCFA	46.816 ± 0.465[c]	48.209 ± 0.400[b]	49.704 ± 0.401[a]	**< 0.001**
Total C18:1	17.776 ± 0.313[a]	16.702 ± 0.270[b]	16.010 ± 0.271[c]	**< 0.001**
Total C18:2	3.002 ± 0.080[a]	2.795 ± 0.069[b]	2.620 ± 0.069[c]	**< 0.001**
Total C18:3	0.910 ± 0.021[a]	0.857 ± 0.018[b]	0.821 ± 0.018[c]	**< 0.001**
Omega 3	1.038 ± 0.021[a]	0.993 ± 0.019[b]	0.965 ± 0.019[b]	**< 0.001**
Omega 6	0.892 ± 0.016[a]	0.838 ± 0.014[b]	0.807 ± 0.014[c]	**< 0.001**
MUFA	21.183 ± 0.329[a]	20.237 ± 0.284[b]	19.547 ± 0.284[c]	**< 0.001**
PUFA	4.184 ± 0.084[a]	3.927 ± 0.073[b]	3.921 ± 0.073[c]	**< 0.001**
Total branched FA	1.620 ± 0.028[a]	1.610 ± 0.024[a]	1.555 ± 0.024[b]	**0.001**
Total UFA	25.367 ± 0.387[a]	24.164 ± 0.334[b]	23.268 ± 0.334[c]	**< 0.001**
Total SFA	71.021 ± 0.423[c]	72.308 ± 0.365[b]	73.298 ± 0.366[a]	**< 0.001**
Total index	26.326 ± 0.410[a]	25.050 ± 0.353[b]	24.098 ± 0.354[c]	**< 0.001**
C18 index	67.597 ± 0.642[a]	65.903 ± 0.553[b]	64.771 ± 0.554[c]	**< 0.001**
CLA index	28.575 ± 0.518[a]	26.828 ± 0.446[b]	25.771 ± 0.447[c]	**< 0.001**

[1] Predicted means and standard error of those means were derived from GLMM. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted as fixed effects. Values within a row with different superscripts differ at $P < 0.05$.

3.3. Discussion

The largest goods export from New Zealand by value is dairy product (John, 2017), and the main product exported was dried milk solids. In a pasture-based system, the ideal cow would therefore be an efficient converter of pasture to milk that contains high levels of these solids. Prior to 2005 pure-bred Jersey and Holstein-Friesian cows were the dominant cattle in the dairy industry, but since then the Holstein-Friesian × Jersey cross-bred (or Kiwicross^{TM}) cow has been developed. With the exception of whole milk production levels, Rowarth (2013) describes this cross as having the best attributes of both Holstein-Friesian and Jersey cows, including having reduced cow size (requiring a lower maintenance cost, and causing less impact on soil structure), being more fertile, having improved calving ease and being longer-lived. As a consequence, data from the 2016-2017 season (LIC) reveals that 48% of cows in NZ are now crosses of varying proportion.

After investigating the promoter region, downstream regions, 12 introns and 9 exons in Kiwicross^{TM} cows, the only variation found in *DGAT1* was the substitution in exon 8 that underpins p.K232A. Other reported variants of *DGAT1* were not found here, including the insertion-deletion variation in the downstream region, the VNTRs in the promoter region (Kuhn *et al.*, 2004), and c.1303A>C in exon 16 (Klaus *et al.*, 2015). This may be because all the cows investigated here were inbred, or not representative of diversity in the Holstein-Friesian or Jersey breeds as a consequence of originating from a single farm, but that does seem unlikely. This would suggest that more Kiwicross^{TM} cows from different farms should be analysed, if not more dairy cows of the other less common breeds found in NZ, such as milking Shorthorns, Guernsey, Brown Swiss and Meuse Rhine Issel cattle. If more cows from different farms were investigated, the previously reported variations of *DGAT1* might be found, along with new variations too.

It has been reported previously that p.K232A affects the milk fat levels and milk yield in different breeds of dairy cattle. For example, as separate breeds, both Holstein-Friesian and Jersey cows with variant K produce more milk fat (Grisart *et al.*, 2002; Mao *et al.*, 2012; Signorelli *et al.*, 2009; Strzalkowska *et al.*, 2005; Tabaran *et al.*, 2015). The frequency (0.619) of K in the Kiwicross^{TM} cows described here was closer to the high end of the previously reported frequency in NZ Holstein-Friesian cows (0.36 to 0.60) (Grisart *et al.*, 2002; Spelman *et al.*, 2002), but not as high as reported previously for NZ Jersey cattle (0.88) (Spelman *et al.*, 2002). Based on New Zealand Breeding Worth (BW) system, Spelman *et al.* (2002) reported that their Q allele (equal to the K variant) provided a selective advantage in NZ cows, while Grisart *et al.* (2002) suggested the K variant could be rapidly incorporated into

cattle using artificial insemination. Given our finding here that *DGAT1* K was associated with increased milk protein and fat content, but reduced milk volume, the benefit of breeding for increased occurrence of K would therefore seem sensible, especially given the positive weighting placed on milk fat percentage and protein percentage traits in the BW evaluation system, and the negative weighting placed on milk volume.

Tabaran *et al.* (2015) have suggested that for Romanian Holstein and Buffalo cows over days 63 to 263 of milking (63 – 263 DIM), higher SFA concentrations in milk, such as for C10:0, C12:0, C15:0, C16:0 and C18:0, could be related to fixation of the K allele. Equally, Duchemin *et al.* (2013) identified that Dutch Holstein-Friesian cows with the K variant produce more C6:0, C8:0 and C16:0 in winter (DIM = 63 to 282) and summer (DIM = 97 to 335), and that the interaction between *DGAT1* genotypes and season were not significant for C16:0 levels.

Bovenhuis *et al.* (2016) investigated the effects of p.K232A on milk fatty acid composition in middle and late lactation Danish Holstein-Friesian cattle (DIM = 129 to 229), Danish Jersey cattle (DIM = 130 to 252) and Dutch Holstein-Friesian cattle (DIM = 63 to 282). The effect of p.K232A on milk saturated FA levels was different in these three breeds, but the cows with the K variant contained more C16:0 in their milk fat. These studies are comparable with what was observed here for Kiwicross^{TM} cows for DIM = 148 ± 19 days, with there being more C6:0, C11:0, C13:0 and C16:0 in the AK and KK cows.

With pasture-based grazing systems, it has been suggested that dairy cattle produce more unsaturated FA and less saturated FA. Chilliard *et al.* (2007) and Coppa *et al.* (2015) revealed that when more fresh herbage was fed, cows produced less C16:0, but more C18:3 n-3, C18:1 *trans*-11 and *cis*-9, *trans*-11 CLA. Equally, when comparing the effects on milk FA composition, of feeding Holstein-Friesian cows (DIM = 192 ± 87) with grass silage versus corn silage-based diets, Van Gastelen *et al.* (2015) revealed that the cows on the grass silage-based diet (which contained more neutral detergent fibre, acid detergent fibre and reducing sugars, but less starch and fat) produced more CLA *cis*-9, *trans*-11 and C18:2 n-3 in the milk.

All the cows in this study were grazed outdoors on mixed perennial ryegrass and white clover pasture. Heck *et al.* (2009) reported a CLA level of 0.76 g/100 g (in August - summer) and C18:3 *cis*-9, 12, 15 FA level of 0.57 g/100 g (in June) in Dutch Holstein-Friesian cows. In comparison Table 3.3 reveals that the cows investigated here produced more *cis*-9, *trans*-11 CLA (0.968 ± 0.016 g/100 g) and C18:3 *cis*-9, 12, 15 FA (0.817 ± 0.005 g/100 g) than the Dutch cows. Capuano *et al.* (2014) described lower CLA levels (0.38 g/100 g) and C18:3 n-3

FA levels (0.52 g/100 g) in cows that were indoors with no fresh grass in their diet, and in Danish Jersey and Holstein cows and Dutch Holstein cows, Bovenhuis *et al.* (2016) found that the content of CLA in these three groups were 0.46 ± 0.11 g/100 g, 0.62 ± 0.16 g/100 g and 0.39 ± 0.11 g/100 g respectively; while the content of C18:3 *cis*-9, 12, 15 FA was 0.41 ± 0.08 g/100 g, 0.49 ± 0.10 g/100 g and 0.41 ± 0.11 g/100 g respectively. It could be concluded that outdoor grazing on pasture is of benefit to increasing CLA and C18:3 *cis*-9, 12, 15 levels.

Compared with pasture feed cows that have been described in previous studies, more *cis*-9, *trans*-11 CLA and C18:3 *cis*-9, 12, 15 FA were produced in the Kiwicross^{TM} cows studied here. For example, the content of *cis*-9, *trans*-11 CLA in Australian cows was 0.908 g/100 g in Autumn and 0.898 g/100 g in Spring (Dunshea *et al.*, 2008), and the content of CLA and C18:3 n-3 was 0.76 g/100 g and 0.68 g/100 g respectively in cows that spent at least 19 hours outdoors on pasture per day (Capuano *et al.*, (2014). Villeneuve *et al.* (2013) investigated Canadian Holstein cows (DIM = 209 ± 53) that were fed with pasture and a concentrate mix (contained 23.3% rolled barley, 23.3% cracked corn, 46.6% soybean meal, as well as 6.7% minerals and vitamins). The content of CLA and C18:3 n-3 in their cow's milk was 0.837 g/100 g and 0.568 g/100 g respectively.

It is notable that the pasture-based diet didn't lead to an increase in all unsaturated FAs. The average concentration of C18:2 *cis*-9, 12 reported here (0.703 ± 0.004 g/100 g), was lower than the concentrations reported by Heck *et al.* (2009) (approximately 1.28 g/100 g), Coppa *et al.* (2015) (1.42–1.43 g/100 g) and Bovenhuis *et al.* (2016) (Danish Jersey: 1.52 ± 0.27 g/100 g; Danish Holstein: 1.69 ± 0.29 g/100 g and Dutch Holstein: 1.20 ± 0.29 g/100 g). Furthermore, an effect of p.K232A on C18:2 *cis*-9, 12 levels was reported by Bovenhuis *et al.* (2016), but was not detected here. This suggests the effects of diet and genetics on C18:2 *cis*-9, 12 levels, might also potentially be a consequence of other factors such as the sampling season, breed, or the stage of lactation.

Heck *et al.* (2009) described differences in milk FA composition in seasonal versus non-seasonal dairy farming systems. The concentrations of C16:1 *trans*-9, C18:1 *trans*-11, and C18:2 *cis*-9, *trans*-11 (CLA) were twice as high in summer milk (pasture grazing from April through to September), than in winter milk, when there was more concentrate in the cows' diets. For example, the *cis*-9, *trans*-11 CLA level reported by Heck *et al.* (2009) was 0.76 g/100 g in summer (August) and 0.38 g/100 g in winter (February). In their study, the effect of season on C18:2 *cis*-9, 12 was significant ($P < 0.001$). During the grass grazing period, the highest and lowest proportion of C18:2 *cis*-9, 12 were in May and September respectively. In NZ, dairy production is predominantly based on the use of pasture, and with a seasonal

calving system. Auldist *et al.* (1998) observed different seasonal patterns to Heck *et al.* (2009), where milk polyunsaturated FA levels (including CLA), were highest in the NZ spring (September). The milk *cis*-9, *trans*-11 CLA level in September reached 0.97–1.27 g/100 g. Dunshea *et al.* (2008) also found that the highest level of milk CLA in Australian dairy herds (calving patterns were non-seasonal) occurred between August and October.

In general, the associations between *DGAT1* p.K232A and the levels of milk FAs described here, especially for C16:0, CLA and C18:3 *cis*-9, 12, 15, were consistent with what has been reported in other breeds. For example, the effects of p.K232A described by Bovenhuis *et al.* (2016) were similar to our finding, with cows with the K variant producing less CLA and C18:3 *cis*-9, 12, 15, but more C16:0 than the cows with the A variant. However, some exceptions do exist in the literature. Juhlin *et al.* (2012) reported that there were no significant difference on the most milk FA levels, between AA and AK cows, the exception being for C16:0 and CLA levels. Similarly, Carvajal *et al.* (2016) did not find any associations between p.K232A and C16:0, C16:1, C18:1, CLA and MUFA levels. The cows that Carvajal *et al.* (2016) investigated were predominantly fed pasture (supplemented with conserved forage such as silage, hay, and maize during autumn and winter). One possible explanation for this inconsistency, was that the cows investigated by Carvajal *et al.* (2016) and Juhlin *et al.* (2012), had milk samples collected over a full year, when the effects of p.K232A on milk FAs described by Duchemin *et al.* (2013), Tabaran *et al.* (2015), Bovenhuis *et al.* (2016) and here, were from the cows sampled after the 60th day in milk.

Some studies suggest that the effects of key genes change during lactation, and that this was caused by variation in their expression (Bionaz *et al.*, 2008b; Bionaz *et al.*, 2012; Wickramasinghe *et al.*, 2012; Yuanyuan *et al.*, 2013). For example, Bionaz *et al.* (2008b) reported that the highest expression of *DGAT1* was at day 15 of lactation and that it decreased markedly in middle and late lactation. Equally, *DGAT1* may interact with other genes. Bionaz *et al.* (2008b), Yuanyuan *et al.* (2013), Wickramasinghe *et al.* (2012) and Bionaz *et al.* (2012) reported the relative contributions of *DGAT1* and the closely related *DGAT2* on milk FA synthesis change during lactation, while Schennink *et al.* (2008) detailed how the effects of *DGAT1* and the Stearoyl-CoA Desaturase gene (*SCD1*) were additive on C16, C18, and CLA levels. Both *DGAT2* and *SCD1* are genes that directly affect bovine milk fat synthesis. Taken together, it could be concluded that while *DGAT1* p.K232A is having an effect on FA levels in the Kiwicross^{TM} cows described in this study, more research is needed to ascertain when and where *DGAT1* is expressed, how it might interact with other genes, and how the epigenetic effects of nutrition and the stage of lactation may moderate the gene's effect on milk traits.

Chapter 4 Variation in *FABP4* and its association with milk traits and milk fat composition

Fatty acid binding protein 4 (FABP4) is a member of the FABP family (FABP1-FABP9), a group of intra-cellular lipid-binding proteins (Zimmerman *et al.*, 1998). The main function of FABP4 is to bind long-chain fatty acids (LCFA) and transport them within animal cells, including mammary gland cells. Previous studies have demonstrated that FABP4 plays a key role in fatty acid (FA) uptake processes in animals (Bionaz *et al.*, 2008b). In the bovine lactation cycle, expression of the FABP4 gene (*FABP4*) was up-regulated during the first 60 days of lactation, when body lipid mobilisation was occurring (Bionaz *et al.*, 2008a). Although the expression of *FABP4* declined subsequently, its expression was still much higher than in non-lactating dairy cows. *FABP4* has therefore been proposed as a candidate gene-marker for milk production traits and milk FA composition (Khatkar *et al.*, 2004).

The FABP4 gene had been mapped to bovine chromosome 14 (BTA 14), and in a region that is rich in quantitative trait loci (QTL) for intramuscular fat content (Michal *et al.*, 2006), milk production traits (Khatkar *et al.*, 2004), and milk FA components (Pegolo *et al.*, 2016). The FABP4 gene has four exons and three introns, and nucleotide sequence variation in the gene has been described in beef cattle (Cho *et al.*, 2008; Williams *et al.*, 2009) and dairy cattle (Nafikov *et al.*, 2013).

Five conserved regions have been described in the promoter region of *FABP4* in cattle, and three of these regions are highly conserved across different animal species (Shin *et al.*, 2009). This suggests they are important cis-regulatory elements and directly involved in regulating *FABP4* gene expression. These include binding sites for C/EBP, activation protein-1, fat-specific enhancer 1 (FSE1), and the CAAT box and TATA box (Figure 4.1). The conserved nature of these elements, suggests that nucleotide sequence variation there-in, may change the expression of *FABP4*, and thus potentially under-pin phenotypic in key fat traits.

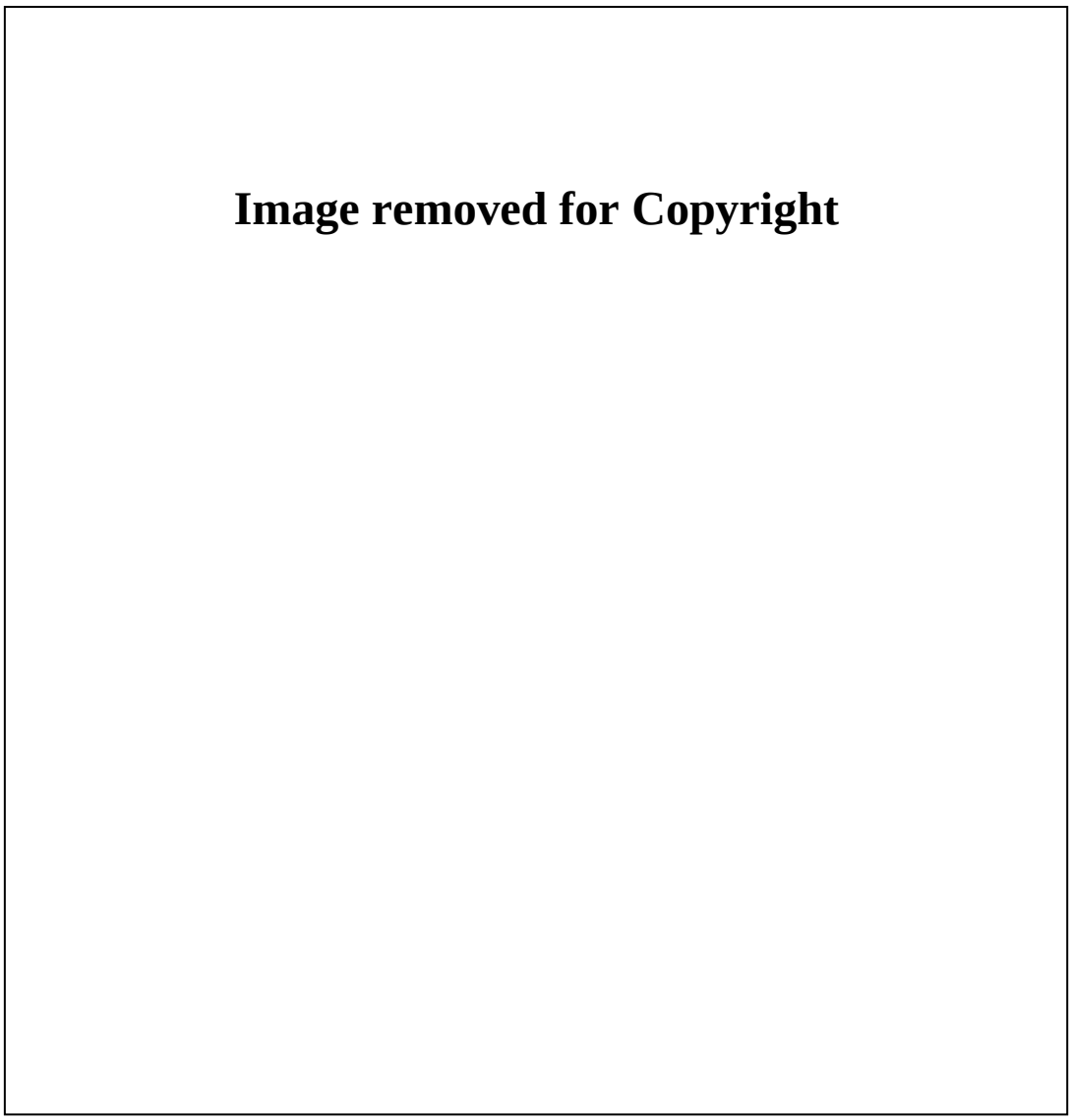


Figure 4. 1 Sequence alignment of the fatty acid-binding protein 4 gene (*FABP4*) promoters of five mammals, and the reported putative transcription binding sites in those promoters (Shin et al., 2009). The abbreviations under the dotted boxes indicate the binding sites for the myocyte-specific enhancer-binding factor (MEF-2); C/EBPα, C/EBPβ, or C/EBPδ; the activation protein-1 (AP-1); the fat-specific enhancer 1 (FSE1); the CAAT box; and the TATA box. The black arrow identifies the ATG codon in all five mammals.

Various effects of *FABP4* variation on other cattle production traits have also been reported. For example it has been associated with meat fat content and FA composition in beef cattle (Michal et al., 2006), and Cho *et al.* (2008) identified 15 nucleotide sequence variations in *FABP4* in native Korean cattle, and two substitutions (c.220A>G and c.349-161T>C, located in exon 2 and intron 3 respectively) were associated with back-fat thickness. However in the same study, the sequence variation c.-259A>G, c.-287A>G, c.74-157A>T in the promoter and intron 1 region, were not associated with back fat thickness. A later paper (Matsumoto *et al.*, 2014) described how c.-287A>G (described as g.-295A>G in their study) can effect carcass traits and fatty acid composition. Additionally, sequence variation at position c.-87T>C of the human *FABP4* promoter region has been demonstrated to lead to reduced C/EBP activity, resulting in reduced transcription of *FABP4* (Tuncman *et al.*, 2006). It could therefore be argued that promotor region variation in *FABP4* needs to be better understood.

Taken together, this supports the contention that more investigation of sequence variation in *FABP4* is needed and in different breeds of cattle, and that its effect on production traits needs to be better understand. Consequently the first objective in this chapter was to search for further variation in the upstream regions of *FABP4*, and if new variation was found in dairy cattle, then its effect on milk traits will be assessed.

In NZ Holstein-Friesian × Jersey (HF×J) - cross dairy cows, Zhou *et al.* (2015) revealed nucleotide sequence variation in the exon 3 and intron 3 region, and identified five sequence variations (three intronic substitutions, one splice-site substitution and one non-synonymous substitution in the exon). Furthermore, they described three *FABP4* haplotypes (*A*, *B* and *C*), and how haplotypes *A* and *B* (differentiated by the non-synonymous substitution c.328G>A) were associated with variation in some gross milk traits. For example, an increase in milk protein percentage and milk fat percentage was found when haplotype *A* was present, while a decrease in milk yield was associated with this haplotypes presence. Zhou *et al.* (2015) did not investigate the effect of this genetic variation on milk FA composition, despite earlier work suggesting that variation in *FABP4* affects milk fat traits (Marchitelli *et al.*, 2013; Nafikov *et al.*, 2013).

Marchitelli *et al.* (2013) demonstrated that c.328G>A in exon 3 of *FABP4* was associated with the medium and long chain FA composition of early lactation milk in Jersey, Piedmontese and Valdostana cows, while Nafikov *et al.* (2013) suggested that c.328G>A affected the C12:0 and monounsaturated FA levels in early lactation milk. Further, Nafikov *et al.* (2013) suggested that once cows had passed through early lactation, the effects of *FABP4* on milk FA composition did not persist into later lactation. This possibly reflects changes in

body fat mobilisation and energy balance at different stages of lactation (e.g. a negative energy balance existing in early lactation) (Maurice-Van Eijndhoven *et al.*, 2011; Soyeurt *et al.*, 2006).

Various studies (including the previous chapter) have revealed other genes that are associated with variation in milk fatty acid composition. Of these, the amino acid substitution p.K232A in *DGAT1* (Grisart *et al.*, 2002; Schennink *et al.*, 2008; Schennink *et al.*, 2007), which is co-located on BTA 14 with *FABP4* is a candidate. Accordingly in this chapter, associations between *FABP4* variation and milk FA levels were investigated in cows that were also genotyped for *DGAT1* p.K232A variation. What-is-more, the current literature describing the likely role of *FABP4* in milk FA production, describes predominantly housed dairy cows (Marchitelli *et al.*, 2013; Nafikov *et al.*, 2013), but these results may not be able to be extrapolated to pasture-based dairy production systems, with cows that are potentially different genetically, by way of having a different genetic background. The objective of this study was therefore to investigate the relationships between *FABP4* and Kiwicross^{TM} cows' milk FA composition, in late lactation, in a wholly pasture-based out-door dairy production system, and in the context of *DGAT1* p.K232A variation.

4.1. Materials and methods

4.1.1. Animals and milk sample collection

The Lincoln University Animal Ethics Committee (AEC Number 521) approved this research under the provisions of the Animal Welfare Act 1999 (NZ Government).

The same cows described in the Chapters 3 were investigated here. A total of 407 Holstein-Friesian × Jersey (HF×J) - cross (Kiwicross^{TM}) dairy cows of 3 to 10 years of age, and from two herds (113 cows in herd 1, 294 cows in herd 2) were investigated. All the cows were grazed on pasture (a mixture of perennial ryegrass and white clover) on the Lincoln University Dairy Farm (LUDF; Canterbury, NZ). All the cows calved over the months August-September, and they were milked twice a day.

Blood samples were collected using the methods described in Chapters 3. The blood samples were analysed at the Lincoln University Gene-Marker Laboratory. A two-step washing procedure (Zhou et al., 2006) was used to purify the genomic DNA.

Samples for milk trait analyses were collected once a month from September 2013 to February 2014. The daily milk yield in litres was recorded using Tru-test milk meters (Tru-test Ltd, Auckland, NZ). These samples were analysed for fat percentage (%) and protein percentage (%) using Fourier-Transform Infra-Red Spectroscopy (MilkoScan FT 120 Foss, Hillerød, Denmark). The milk samples for FA analysis were collected from each cow in a single afternoon milking on 15th January 2014 (days in milk (DIM) = 148 ± 19 days). These were frozen at -20 ^{o}C, and then freeze-dried, prior to being individually ground to a fine powder for component analysis. As in Chapter 3, DNA samples (n = 25) were used to develop a PCR-SSCP protocol for each of the four regions of dairy cattle *FABP4* to be investigated.

4.1.2. Gas Chromatography of the Fatty Acids in the Milk Sample

Gas Chromatography of the fatty acids in the milk samples was as described in Chapters 3.

4.1.3. PCR primers used for dairy cattle *FABP4* amplification

Four sets of primers (Table 4.1) were designed to amplify four regions of *FABP4*, based on the cattle reference sequence, Reference sequence number: GY146938.1. Region 1, 2, 3 and 4 spanned the promotor, which covered the previously reported nucleotide sequence variation including c.-295A>G and c.-287A>G reported by Cho *et al.* (2008) that associated with back fat thickness. Region 4 also spanned the 5'UTR, exon 1 and part of intron 1 (Figure 4.2). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Table 4. 1 PCR primers used to amplify four regions of the dairy cattle FABP4 gene (*FABP4*).

***FABP4* region[1]**	**Amplicon size (bp)**	**Forward primer**	**Reverse primer**
1	510	5'-ATCATGAGTTCCTTATTGCC-3'	5'-CATAGACATATTATAGACTGTG-3'
2	590	5'-GATGAATTCATAATAGTAAGC-3'	5'-ATTCGTCCTGTTCCTGAC-3'
3	544	5'-TAGACCTCAGAAGCTTGTGAC-3'	5'-TATTTATTCCAGTGTAGAGTG-3'
4	543	5'-ACACTGGGTCCACTCTACAC-3'	5'-CAGACCAATGCTTCCAGAGTG-3'

[1] See Figure 4.2

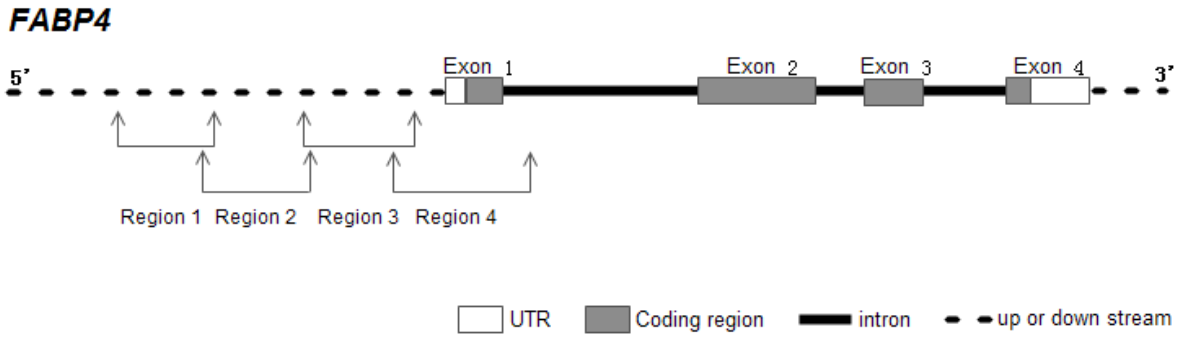


Figure 4. 2 Location of *FABP4* regions that were amplified. Four sets of primers were designed in order to amplify a 510 bp, 590 bp and 544 bp region (1, 2 and 3) in the promotor, and a 543 bp region spanning the promotor to intron 1. The gene structure is based on the cattle *FABP4* sequence and is not drawn to scale. Cattle *FABP4* has 4 exons long (Reference sequence number: GY146938.1).

4.1.4. Developing the PCR-SSCP protocol for *FABP4*

DNA samples (n = 25) were used to develop the PCR-SSCP protocols for analysis of the target regions of *FABP4*. The PCR protocols were optimised with different annealing temperatures (between 50 ^{o}C to 62 ^{o}C). Electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels was used to visualise the target amplicons, with 1× TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide being used.

When the agarose gels produced a satisfactory result, the conditions for band separation and resolution were optimised at different percentage of acrylamide in SSCP gels (10 %, 12 % and 14 %), different glycerol concentrations (0.5% - 4%) and at various temperatures (3 ^{o}C – 35 ^{o}C). Amplicons were loaded onto 16 cm x 18 cm, 14% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 250 V for 19 hours at 15 ^{o}C in 0.5× TBE buffer.

4.1.5. PCR analysis and genotyping of dairy cattle *FABP4*

Blood samples were collected using the methods described in Chapters 3. The blood samples were analysed at the Lincoln University Gene-Marker laboratory. A two-step washing procedure (Zhou *et al.*, 2006) was used to purify genomic DNA.

PCR amplification were performed in a 15-µL reaction containing the genomic DNA (punch of FTA paper), 0.25 µM of each designed primer, 150 µM of each dNTP (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the polymerase enzyme.

Amplification was undertaken using S1000 thermal cyclers (Bio-Red, Hercules, CA, USA) and the thermal profile included an initial denaturation for 2 minutes at 94 ^{o}C; followed by 35 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 58 ^{o}C and 30 seconds at 72 ^{o}C; with a final extension for 5 minutes at 72 ^{o}C. Following amplification, a 0.7-µL aliquot of the PCR products was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ^{o}C for 5 minutes and rapid cooling on wet ice, the samples were loaded on 16 cm × 18 cm, acrylamide: bisacrylamide (37.5: 1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) in 0.5× TBE buffer. The method of Byun *et al.* (2009) was used to silver-stain the gels.

For Region 3, the best annealing temperature was 56 ^{o}C. The Region 3 amplicons were loaded onto 16 cm × 18 cm, 12% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 350 V for 19 hours at 20 ^{o}C in 0.5× TBE buffer.

4.1.6. Haplotypes of the dairy cattle *FABP4* analysed

The three *FABP4* haplotypes (*A, B* and *C*) reported by Zhou *et al.* (2015) are listed in Figure 4.3 The association between the three haplotypes and milk fat composition were investigated.

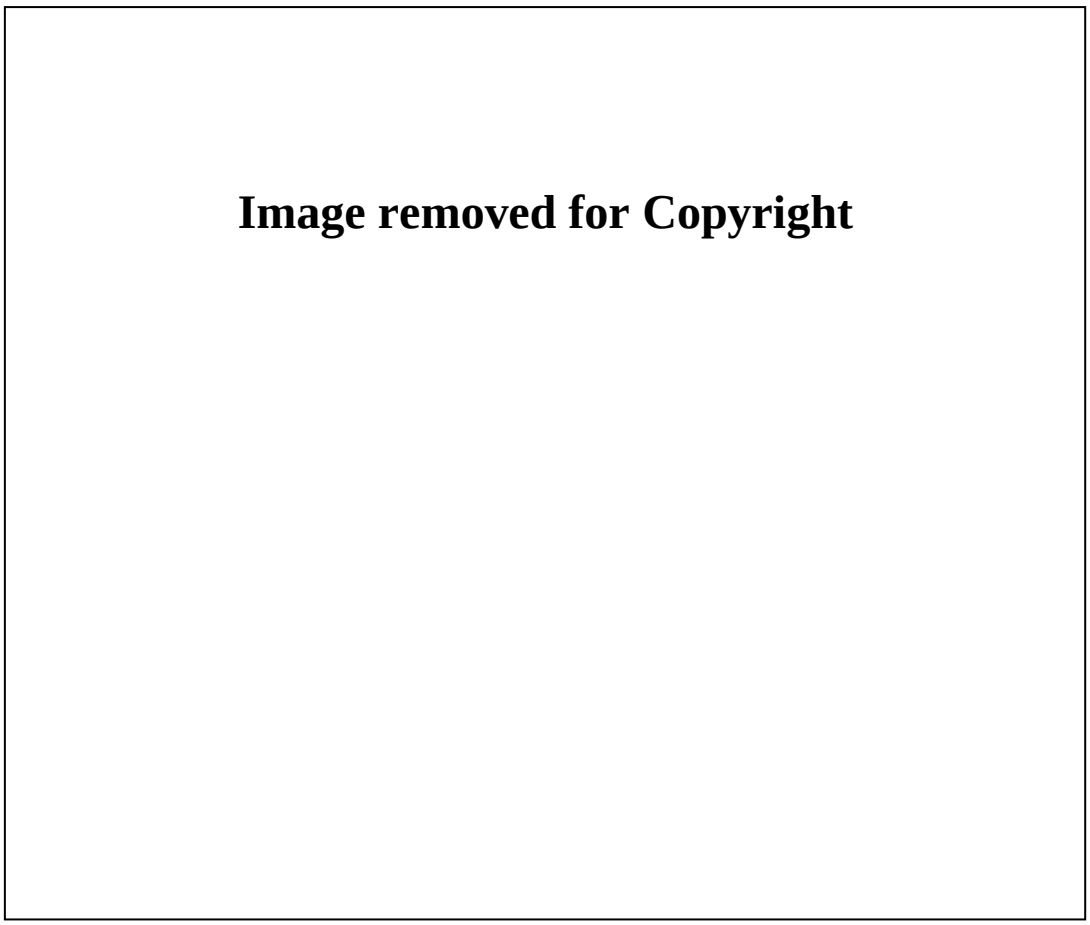


Figure 4. 3 Variation in the bovine FABP4 gene (*FABP4*) (Zhou *et al.*, 2015). (a) Three PCR-SSCP banding patterns representing. (b) Three haplotype sequences (*A*, *B* and *C*) were detected in the exon 3–intron 3 region. (c) Of the five SNPs identified, two were located in exon 3 and three were in intron 3 (Zhou *et al.*, 2015).

4.1.7. Statistical analysis

Hardy-Weinberg equilibrium (HWE) for the *FABP4* genotypes was analysed using an online chi-square calculator (http://www.oege.org/software/hwe-mr-calc.shtml).

All statistical analysis was carried out using IBM SPSS version 22 (IBM, NY, USA). Associations between variation in *FABP4* and variation in milk fatty acid traits were tested using General Linear Mixed-effects Models (GLMMs). As some measurements were made in percentages, a gamma regression function was adopted in the GLMMs. Single-haplotype presence/absence models (fixed effects: DIM, age, herd and *DGAT1* p.K232A type) were used to ascertain which haplotypes should be analysed in subsequent multi-haplotype models. The multi-haplotype models included any haplotype that had a haplotype-fatty acid trait

association in the single-haplotype presence/absence analysis with a *P*-value of less than 0.200, and which might therefore affect the trait. The multi-haplotype models were again corrected for the fixed effects of (DIM, age, herd and *DGAT1* p.K232A) and with haplotype fitted as a random effect. A GLMM (fixed effect: genotype, DIM, age, herd and *DGAT1* p.K232A) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes with a frequency greater than 5% (thus insuring adequate sample size), on milk FA traits.

The effect of cow sire could not be included in the GLMMs. Some semen straws (sire genetics) used in NZ dairy cattle artificial insemination breeding, contain mixed-sire semen purchased from commercial semen producers. In these cases, individual sire identity is impossible to ascertain, but because the straws were mixed-semen straws and because different sires are used for different inseminations in different years, it is unlikely that sire was a strongly confounding effect. Cow age and herd might also therefore be confounded with sire, but this cannot be confirmed.

4.2. Results

4.2.1. PCR-SSCP typing of *FABP4*

The sequence of Region 1, 2 and 4 didn't show any variations in the 407 cows tested. Sequence variation was found in the Region 3, and three genotypes were identified ($P_{AA,}$ P_{BB} and P_{AB}; Figure 4.4).

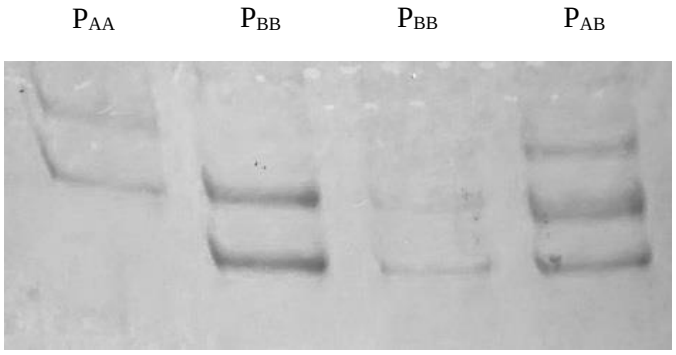


Figure 4. 4 PCR-SSCP of the promoter region of *FABP4*. Two banding patterns (P_A and P_B) were detected in Region 3.

4.2.2. Milk production traits, fatty acid composition and *FABP4* variation

The results of milk fatty acid analysis by GC are shown in Chapter 3. The effects of the variation in *FABP4* Region 3 ($P_{AA,}$ P_{BB} and P_{AB}) on milk traits and milk fat composition were not significant, and hence the results of the association analysis are not shown. The presence or absence of haplotypes *A*, *B* and *C*, identified in the exon 3 and intron 3 (Figure 4.3), did affect milk fat composition.

Table 4.2 and 4.3 summarise the associations revealed between the *FABP4* haplotypes, and the composition of individual and grouped FAs respectively. Results are not presented if an association was not found. The predicted means from the models and standard errors of the means derived from the GLMMs were very similar for the single-haplotype model and multi-haplotype model, with only minor differences occurring for the C10:1, C14:0 and C22:0 FA results. The p.K232A substitution of *DGAT1* affected many of the milk fatty acid levels in these animals. Significant effects of p.K232A (as a fixed factor in the model) on many FAs are illustrated in Table 4.2 and 4.3. Both *DGAT1* and *FABP4* affected the levels MCFA. At the level of individual FAs, only C14:0, C15:0 *iso* and C18:2 *cis*-9, 12 FA were affected by these two genes.

Table 4. 2 Association between grouped fatty acid levels and *FABP4* variation

FAME	Haplotype	Other haplotype in model	Mean FAME level ± SE[1] (g/100 g milk FA) associated with *FABP4* haplotypes				*P* value[2]	
			Absent	n	Present	n	*DGAT1*	*FABP4*
MCFA	*A*	none	19.583 ± 0.253	100	19.696 ± 0.212	307	**< 0.001**	0.534
	B	none	19.490 ± 0.215	280	19.980 ± 0.230	127	**0.001**	**0.003**
	C	none	19.814 ± 0.221	182	19.528 ± 0.221	225	**< 0.001**	*0.062*
	B	*C*	19.490 ± 0.215	280	19.980 ± 0.230	127	**0.001**	**0.003**
	C	*B*	19.807 ± 0.297	182	19.630 ± 0.301	225	**0.001**	0.268
Omega6 FAs	*A*	none	0.829 ± 0.014	100	0.835 ± 0.012	307	**< 0.001**	0.514
	B	none	0.841 ± 0.012	280	0.822 ± 0.013	127	**< 0.001**	**0.040**
	C	none	0.834 ± 0.012	182	0.833 ± 0.012	225	**< 0.001**	0.948
Total branched	*A*	none	1.595 ± 0.025	100	1.585 ± 0.021	307	**0.004**	0.588
	B	none	1.575 ± 0.021	280	1.608 ± 0.023	127	**0.004**	**0.042**
	C	none	1.596 ± 0.022	182	1.578 ± 0.022	225	**0.003**	0.24
C12:1 index	*A*	none	2.248 ± 0.059	100	2.256 ± 0.050	307	0.447	0.855
	B	none	2.231 ± 0.051	280	2.293 ± 0.054	127	0.389	*0.113*
	C	none	2.294 ± 0.052	182	2.213 ± 0.052	225	0.409	**0.023**
	B	*C*	2.237 ± 0.059	280	2.281 ± 0.062	127	0.386	0.272
	C	*B*	2.294 ± 0.052	182	2.212 ± 0.052	225	0.408	**0.023**
CLA index	*A*	none	26.976 ± 0.456	100	27.156 ± 0.380	307	**< 0.001**	0.581
	B	none	27.007 ± 0.390	280	27.308 ± 0.418	127	**< 0.001**	0.312
	C	none	27.403 ± 0.396	182	26.827 ± 0.398	225	**<0.001**	**0.037**

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)', 'herd' and '*DGAT1* p.K232A' were fitted to the models as fixed effects.
[2] $0.05 < P < 0.2$ in italics; $P < 0.05$ in bold.

Table 4. 3 Association between individual fatty acid levels and *FABP4* variation

FAME	Haplotype	Other haplotype in model	Mean FAME level ± SE[1] (g/100 g milk FA) associated with *FABP4* haplotypes				*P* value[2]	
			Absent	n	Present	n	*DGAT1*	*FABP4*
C8:0	*A*	none	1.178 ± 0.015	100	1.178 ± 0.013	307	*0.127*	0.996
	B	none	1.171 ± 0.013	280	1.191 ± 0.014	127	*0.094*	**0.040**
	C	none	1.184 ± 0.013	182	1.173 ± 0.013	225	*0.125*	0.247
C10:0	*A*	none	3.218 ± 0.060	100	3.222 ± 0.050	307	0.532	0.916
	B	none	3.185 ± 0.051	280	3.283 ± 0.055	127	0.481	**0.012**
	C	none	3.244 ± 0.052	182	3.199 ± 0.053	225	0.497	0.217
C10:1	*A*	none	0.280 ± 0.007	100	0.280 ± 0.006	307	0.221	0.932
	B	none	0.277 ± 0.006	280	0.287 ± 0.007	127	*0.180*	**0.039**
	C	none	0.286 ± 0.006	182	0.275 ± 0.006	225	*0.185*	**0.010**
	B	*C*	0.277 ± 0.008	280	0.285 ± 0.008	127	*0.169*	*0.136*
	C	*B*	0.286 ± 0.007	182	0.275 ± 0.007	225	*0.176*	**0.019**
C12:0	*A*	none	3.896 ± 0.080	100	3.910 ± 0.067	307	0.552	0.819
	B	none	3.856 ± 0.068	280	3.993 ± 0.073	127	0.501	**0.009**
	C	none	3.945 ± 0.070	182	3.867 ± 0.070	225	0.507	*0.112*
	B	*C*	3.856 ± 0.068	280	3.993 ± 0.073	127	0.500	**0.009**
	C	*B*	3.943 ± 0.089	182	3.895 ± 0.090	225	0.489	0.341
C13:0 *iso*	*A*	none	0.078 ± 0.003	100	0.079 ± 0.002	307	0.202	0.946
	B	none	0.077 ± 0.002	280	0.081 ± 0.002	127	*0.155*	**0.014**
	C	none	0.081 ± 0.002	182	0.076 ± 0.002	225	*0.169*	**0.008**
C12:1	*A*	none	0.090 ± 0.003	100	0.090 ± 0.003	307	0.339	0.891
	B	none	0.088 ± 0.003	280	0.093 ± 0.003	127	0.262	**0.005**
	C	none	0.092 ± 0.003	182	0.087 ± 0.003	225	0.292	**0.008**
	B	*C*	0.088 ± 0.003	280	0.093 ± 0.003	127	0.264	**0.023**
	C	*B*	0.092 ± 0.003	182	0.088 ± 0.003	225	0.270	**0.041**
C14:0	*A*	none	12.469 ± 0.132	100	12.565 ± 0.110	307	**< 0.001**	0.314
	B	none	12.450 ± 0.112	280	12.705 ± 0.120	127	**< 0.001**	**0.003**
	C	none	12.626 ± 0.115	182	12.462 ± 0.115	225	**< 0.001**	**0.041**
	B	*C*	12.453 ± 0.115	280	12.699 ± 0.123	127	**< 0.001**	**0.005**
	C	*B*	12.622 ± 0.153	182	12.513 ± 0.155	225	**< 0.001**	*0.191*
C14:1 *cis*-9	*A*	none	0.946 ± 0.036	100	0.957 ± 0.030	307	0.223	0.658
	B	none	0.940 ± 0.030	280	0.981 ± 0.033	127	0.217	*0.075*
	C	none	0.981 ± 0.031	182	0.929 ± 0.031	225	*0.179*	**0.016**
	B	*C*	0.943 ± 0.036	280	0.974 ± 0.038	127	*0.191*	0.211
	C	*B*	0.980 ± 0.031	182	0.930 ± 0.031	225	*0.180*	**0.019**
C15:0 *iso*	*A*	none	0.284 ± 0.004	100	0.289 ± 0.004	307	**< 0.001**	*0.139*
	B	none	0.286 ± 0.004	280	0.290 ± 0.004	127	**< 0.001**	*0.130*
	C	none	0.291 ± 0.004	182	0.284 ± 0.004	225	**< 0.001**	**0.015**
C17:0 *iso*	*A*	none	0.568 ± 0.011	100	0.553 ± 0.009	307	*0.120*	*0.061*
	B	none	0.550 ± 0.009	280	0.568 ± 0.010	127	*0.100*	**0.013**
	C	none	0.557 ± 0.010	182	0.556 ± 0.010	225	*0.108*	0.935
	A	*B*	0.567 ± 0.013	100	0.556 ± 0.011	307	*0.109*	0.155
	B	*A*	0.551 ± 0.010	280	0.568 ± 0.011	127	*0.102*	**0.020**
C18:2 *cis*-9, 12	*A*	none	0.691 ± 0.013	100	0.697 ± 0.011	307	**< 0.001**	0.548
	B	none	0.702 ± 0.011	280	0.685 ± 0.012	127	**< 0.001**	**0.043**
	C	none	0.696 ± 0.112	182	0.696 ± 0.012	225	**< 0.001**	0.937
C22:0	*A*	none	0.061 ± 0.002	100	0.066 ± 0.002	307	*0.147*	**0.001**
	B	none	0.066 ± 0.002	280	0.063 ± 0.002	127	0.215	**0.031**
	C	none	0.065 ± 0.002	182	0.064 ± 0.002	225	0.255	0.613
	A	*B*	0.061 ± 0.002	100	0.066 ± 0.002	307	*0.144*	**0.002**
	B	*A*	0.064 ± 0.003	280	0.062 ± 0.003	127	*0.147*	*0.141*
C24:0	*A*	none	0.042 ± 0.002	100	0.046 ± 0.001	307	*0.184*	**< 0.001**
	B	none	0.046 ± 0.001	280	0.044 ± 0.001	127	0.289	*0.083*
	C	none	0.045 ± 0.001	182	0.045 ± 0.001	225	0.332	0.532
	A	*B*	0.042 ± 0.002	100	0.046 ± 0.001	307	*0.164*	**< 0.001**
	B	*A*	0.044 ± 0.002	280	0.043 ± 0.002	127	*0.162*	0.370
C22:5	*A*	none	0.121 ± 0.004	100	0.122 ± 0.003	307	0.212	0.794
	B	none	0.124 ± 0.003	280	0.118 ± 0.004	127	0.239	**0.031**
	C	none	0.123 ± 0.003	182	0.121 ± 0.004	225	0.208	0.590

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)', 'herd' and '*DGAT1* p.K232A' were fitted to the models as fixed effects.

[2] $0.05 < P < 0.2$ in italics; $P < 0.05$ in bold.

The presence of *FABP4* haplotype *A* was associated with an increase in long chain saturated FAs (Table 4.3), including the C22:0 and C24:0 FA. The presence of haplotype *B* was associated with an increase in C8:0, C10:0, C10:1, C12:0, C13:0 *iso*, C12:1, C14:0, C17:0 *iso* and total branched FAs. It was also associated with a decrease in C18:2 *cis*-9, 12, C22:0, C22:5 *cis*-7, 10, 13, 16, 19 and Omega6 FAs. The presence of variant *B* was also associated with an increase in the MCFA group, as a consequence of the increase in C10:0, C12:0 and C14:0 FA. The presence of haplotype *C* was associated with a decrease for C10:1, C13:0 *iso*, C12:1, C14:1*cis*-9, C15:0 *iso* FA, C12:1 index and CLA index.

All milk FA traits were analysed at the genotype level with five *FABP4* genotypes (*AA*, *AB*, *AC*, *BC* and *CC*) included in the analysis. These results are summarized in Table 4.4. Due to the low number of *BB* cows (n = 14), this genotype was not included in the analysis. Pairwise comparisons, using Bonferroni corrections, revealed that dairy cattle with genotype *BC* produced significantly less C22:0 FA than *AA*, *AB* and *AC* cows. The genotype *BC* also produced significantly less C24:0 FA than *AC* cows. The dairy cattle with genotype *AB*, produced more C14:0 FA than the dairy cattle with *AA*, *AC* and *CC*. Both the *FABP4* variation and *DGAT1* (p.K232A) were associated with the C14:0 FA levels.

Table 4. 4 Association between milk fatty acid levels and *FABP4* genotypes corrected for *DGAT1* genotype [1]

FAME	Mean ± SE [1] (g/100 g milk FA) associated with *FABP4* genotypes					*P* value	
	AA (n = 96)	***AB*** (n = 74)	***AC*** (n = 137)	***BC*** (n = 41)	***CC*** (n = 47)	***DGAT1***	***FABP4***
C8:0	1.177 ± 0.016	1.195 ± 0.016	1.176 ± 0.015	1.191 ± 0.020	1.167 ± 0.019	**0.032**[3]	0.429
C10:0	3.216 ± 0.063	3.308 ± 0.065	3.211 ± 0.060	3.294 ± 0.077	3.168 ± 0.075	0.342	0.170
C10:1	0.284 ± 0.008	0.285 ± 0.008	0.271 ± 0.007	0.285 ± 0.010	0.270 ± 0.009	0.122	0.062
C12:0	3.907 ± 0.084	4.029 ± 0.086	3.878 ± 0.080	4.000 ± 0.103	3.825 ± 0.100	0.395	0.107
C13:0 *iso*	0.080 ± 0.003	0.081 ± 0.003	0.075 ± 0.003	0.081 ± 0.003	0.075 ± 0.003	0.171	**0.025**
C12:1	0.091 ± 0.003^{ab}	0.094 ± 0.003^{a}	0.087 ± 0.003^{b}	0.093 ± 0.004^{ab}	0.086 ± 0.004^{ab}	0.255	**0.018**
C14:0	12.511 ± 0.137^{b}	12.881 ± 0.142^{a}	12.525 ± 0.132^{b}	12.563 ± 0.169$^{\mathbf{ab}}$	12.435 ± 0.165^{b}	**< 0.001**	**0.010**
C14:1 *cis*-9	0.971 ± 0.037	0.979 ± 0.039	0.912 ± 0.036	0.965 ± 0.046	0.903 ± 0.045	0.155	0.201
C15:0 *iso*	0.291 ± 0.005	0.292 ± 0.005	0.285 ± 0.004	0.289 ± 0.006	0.278 ± 0.006	**< 0.001**	**0.041**
C17:0 *iso*	0.054 ± 0.011	0.563 ± 0.012	0.554 ± 0.011	0.584 ± 0.014	0.557 ± 0.014	0.087	0.120
C18:2 *cis*-9, 12	0.721 ± 0.014	0.687 ± 0.014	0.711 ± 0.013	0.707 ± 0.017	0.699 ± 0.016	< 0.001	0.077
C22:0	0.066 ± 0.002^{a}	0.066 ± 0.002^{a}	0.067 ± 0.002^{a}	0.059 ± 0.003^{b}	0.065 ± 0.003^{ab}	0.085	**0.021**
C24:0	0.045 ± 0.002^{ab}	0.046 ± 0.002^{ab}	0.046 ± 0.002^{a}	0.041 ± 0.002^{b}	0.043 ± 0.002^{ab}	0.096	**0.030**
C22:5 *cis*-7, 10, 13, 16, 19	0.128 ± 0.004	0.120 ± 0.004	0.122 ± 0.004	0.119 ± 0.005	0.127 ± 0.005	0.328	0.121
MCFA	19.634 ± 0.264	20.218 ± 0.272	19.614 ± 0.252	19.857 ± 0.325	19.428 ± 0.317	**0.001**	**0.032**
Omega6 FAs	0.862 ± 0.014	0.826 ± 0.015	0.850 ± 0.014	0.845 ± 0.018	0.838 ± 0.017	**< 0.001**	0.072
Total branched FA	1.602 ± 0.026	1.605 ± 0.027	1.578 ± 0.025	1.639 ± 0.032	1.564 ± 0.031	**0.001**	0.101
C12:1 index	2.284 ± 0.062	2.285 ± 0.065	2.185 ± 0.060	2.274 ± 0.077	2.184 ± 0.075	0.451	0.129
CLA index	27.351 ± 0.480	27.340 ± 0.500	26.733 ± 0.459	26.995 ± 0.591	26.642 ± 0.577	**< 0.001**	0.331

[1] The genotypes with a frequency greater than 5% were analysed. The frequency of BB (n = 14) was 3.07 %.
[2] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)', 'herd' and 'DGAT1 p.K232A' were fitted to the models as fixed effects. Means within a row that do not share a superscript letter are separated by Bonferroni test at P < 0.05
[3] P < 0.05 in bold.

4.3. Discussion

In cows, biosynthetic pathways operating in the mammary glands produce FAs of an even number of carbons in length, these ranging from 4 to 16 carbons long. However C16:0 FA, and the other LCFAs, can also originate from dietary lipid sources and from lipolysis, the breakdown of cow adipose tissue. In this respect, the milk FA profiles described here for pasture-fed dairy cattle are similar to previously reported profiles from NZ (Schwendel *et al.* (2015), especially for samples from late lactation (autumn) milk. Thomson (2000) also described FA profiles in NZ pasture-fed dairy cows, noting how the concentrations of PUFA and CLA in milk fat are highest during spring and autumn in NZ, but decline during summer, this reflecting reduced pasture quality. Specifically, the CLA levels declined from a concentration 1-1.4% through spring, to 1.0% in summer and then increased to 1.6% by April. With the samples in this study being collected in mid-January (mid-summer in NZ), the average CLA level measured here (0.968 ± 0.016 g/100g, Table 3.3), sits comfortably with these observations.

Thomson (2000) also noted how the concentrations of FA groups and individual FAs are similar in spring and autumn on pasture, but differ in summer. For example, in early lactation (August), the ratio of pre-formed to *de-novo* synthesised FAs (the P:S ratio) was 0.76, but this declined to 0.51 by December, then increased to 0.80 by late autumn. The concentration of total UFA followed a similar trend: 30.4% in August, 24.5% in December and 32.8% in April, this also comparing favourably with the average total UFA (23.891 ± 0.118 g/100g, Table 3.4) in this study. Thomson (2000) also noted that in early lactation, milk fat from primiparous cows had higher C18:1, total UFA and P:S ratio, than milk from multiparous cows, this indicating greater mobilisation of body fat. Together this suggests that while the figures reported in this chapter appear to be typical of pasture-fed cows in NZ, care needs to be taken in describing and/or accommodating cows of different parity (or age) and the stage of lactation (or DIM), when describing milk FA profiles in different studies.

In this study, the three *FABP4* haplotypes (*A*, *B* and *C*) identified by Zhou *et al.* (2015) were associated variously with differences in MCFA, LCFA and some UFA levels (Table 4.2 and 4.3). Our previous study described the high frequency of K variant of *DGAT1* in New Zealand dairy cattle whose milk FA composition was changed significantly (Chapter 3). In this study, similar effects of p.K232A were found on the milk FA levels (Table 4.4). Both *DGAT1* p.K232A and the variants of *FABP4* described here affected the MCFA, Omega 6, total branched FAs and CLA index levels, but their effects on the individual FA levels were

different. Only the C14:0, C15:0 *iso* and C18:2 *cis*-9, 12 FA levels were found to be affected by these two genes. That suggests effects of *FABP4* on C8:0, C10:0, C10:1, C12:0, C13:0 *iso*, C12:1, C14:1, C17:0 *iso*, C22:0, C24:0 and C22:5 *cis*-7, 10, 13, 16, 19 FA levels described here were likely independent. Potential interaction effect between *DGAT1* and *FABP4* might exist, because they are all located on BTA14 with a small physical distance (about 44 mega base pairs) between them. After correcting for the influence of *DGAT1*, association between C14:0, C15:0 *iso* and C18:2 *cis*-9, 12 levels and *FABP4* still existed (Table 4.2 and 4.4).

Pegolo *et al.* (2016) described the effects of *FABP4* variation (Reference SNP: 110757796) on C6:0, C18:1 *trans*-4 and C18:2 *cis*-9, 12 levels, while Nafikov *et al.* (2013) reported that *FABP4* variation (Reference SNP: 110652478) affected the levels of C10:0, C12:0, C14:0, SFA, UFA, MUFA and the SFA/UFA ratio. However, while Nafikov *et al.* (2013) described how cows with haplotype H3 (c.328A - equivalent to haplotype *B* here), had reduced milk C10:0, C12:0 and C14:0 levels, the opposite effect of *FABP4* variation were found in this study (see Table 4.2). Only the C14:0 and MCFA levels were affected by both *FABP4* and *DGAT1*.This would suggest that the effect of *FABP4* variation may also be specific to lactation stage (or DIM), cattle breed, feeding system, or a combination of all these factors.

In the context of the stage of lactation or DIM, the cows with haplotype *B* in this study appeared to have a higher milk content of C8:0, C10:0, C12:0 and C14:0 FA in later lactation (DIM = 148 ± 19 days), but the cows with haplotype H3 described by (Nafikov *et al.*, 2013) had a lower concentration of C10:0, C12:0 and C14:0 in early lactation (1 to 90 DIM). Other studies have also reported that the composition of MCFAs changes at different stages of lactation stages. For example, Schwendel *et al.* (2015) describe differences between C10:0, C12:0 and C14:0 levels in two herds with different pasture diets, when comparing cows that were 90-100 DIM, and cows that were 202-212 DIM. Garnsworthy *et al.* (2006) described variation in milk FA composition in early lactation (4 to 29 DIM), mid-lactation (103 to 156 DIM) and late lactation (265 to 306 DIM) in Holstein cows that were fed an identical diet (34% corn silage, 14% grass silage, 17% soybean meal, 15% wheat, 9% brewers' grains, 8% palm kernel meal, 2% rumen-inert fat and 1% Hi-Phos), and revealed that the levels of C10:0, C12:0 and C14:0 were lower for early lactation cows, when compared to the other stages of lactation, although the FA levels were similar when comparing between mid and late lactation cows. Nafikov *et al.* (2013) did not find any association between H3 and medium chain FAs in the later stages of lactation stage (90-300 DIM), but it is possible, and might be consistent with what is reported in this study, that a different effect under-pinned by *FABP4* variation, occurs at different stages of lactation.

Lactation stages might not however affect LCFA levels. Marchitelli *et al.* (2013) reported that their "major" *FABP4* allele, which corresponds to both haplotypes *A* and *B* here, had a positive effect on C18:0 levels and LCFA levels, and that the effect could be observed in Jersey, Piedmontese and Valdostana cows at three different stages of lactation: before the 60th DIM, between 100-140 DIM and around the 210th DIM. The C18:0 FA levels did not change significantly in Piedmontese and Valdostana cows and the LCFA level didn't changed significantly in Jersey cows throughout the lactation. In this study, haplotype *A* (c.328G) also had a positive effect on C22:0 and C24:0 levels at 148 ± 19 DIM. Garnsworthy *et al.* (2006) also reported that the stage of lactation stage didn't affect milk C18:0 and C20:0 levels significantly in non-pasture fed Holstein cows.

The effect of breed differences was not fully interrogated by Marchitelli *et al.* (2013), despite reporting on three breeds; Jersey, Piedmontese and Valdostana cows. The effects of their major allele on C8:0, C10:0, C12:0, C14:0 and C18:1 levels were not significant in all breeds, and possibly because the milk FA composition was different in the different breeds. Maurice-Van Eijndhoven *et al.* (2013) and Soyeurt *et al.* (2006) described significant differences in C10:0, C14:0, C16:0 and C18:0 levels between Holstein-Friesian cows and Jersey cows. Contrastingly though, Palladino *et al.* (2010) reported that the C8:0, C10:0, C12:0, C14:0, C18:0 and LCFA levels were similar when comparing Holstein-Friesian and Jersey dairy cows, and their F1 hybrid. In this context, breed may therefore not be of major consequence in understanding the effect of *FABP4* variation in the H-F×J - cross cows in this study. Marchitelli *et al.* (2013) also described significant positive effects from their major allele on C18:0 and LCFA levels in all three breeds they studied. Taken together, the effect of *FABP4* variation on C20:0, C22:0 and C24:0 FA levels identified in this study, would not appear to be a consequence of, or affected by, breed variation.

An opposite effect of *FABP4* variation on some milk FA levels was found in this study when compared to the results of Nafikov *et al.* (2013), although as described above, the two studies were undertaken at a different stage of lactation. The wholly pasture-based dairy production system described here, is a different feeding system to the system used in the studies of Marchitelli *et al.* (2013) and Nafikov *et al.* (2013). Differences in how the cows in the different studies were fed might therefore lead to the differing results observed.

Some studies suggest that certain FA levels are not changed significantly when diet is changed. For example, Schroeder *et al.* (2003) changed a TMR diet (containing 18% corn grain, 59% corn silage, 22% sunflower meal, 0.5% urea, and 0.9% mineral-vitamin premix on DM basis) to pasture plus 6.7 kg DM/d of a corn-based concentrate in two groups of Holstein

cows. After being on the different diets for five weeks (at 117 ± 6 DIM), they found that there wasn't a difference between these two groups in the levels of C10:0, C12:0 and C14:0 FAs in milk. Equally, Schwendel *et al.* (2015) compared milk FA composition between a conventional pasture-based diet (year-round pasture-based grazing) and an organic diet, where cattle were also fed 10 mg of garlic oil per cow per day, and 18 g fish oil per day. They also found that there wasn't a difference in milk C8:0, C10:0, C12:0 and C14:0 FA levels in the conventional herd (at 100 and 212 DIM) and the organic herd (at 90 and 202 DIM).

Marchitelli *et al.* (2013) fed corn, silage and concentrates to their cows. The effects of *FABP4* variation on milk LCFAs when comparing the results described by Marchitelli *et al.* (2013) with the results of study, might reflect differences in how the cows were fed. However, it's hard to explain which dietary ingredient lead to this difference, because there are seemingly inconsistent results in previous studies. Schwendel *et al.* (2015) describe how milk long chain FAs can be affected by feeding systems, whereas Wales *et al.* (2009) conclude that the C18:0 and C20:0 FA levels in milk do not appear to be affected by the increasing amount of supplementation with concentrate (65% rolled barley grain, 35% steam-flaked corn grain, and 5% molasses) in a pasture-based diet. Furthermore, Rego *et al.* (2016) reported that only C18:0 and C22:0 FA levels change, but that the level of C22:0 and C24:0 FA didn't, when the diet was switched from pasture to a total mixed ration diet, and then back to pasture.

Taken together, while *FABP4* certainly seems to be an important gene as regards milk FA component levels, deciphering its effects in the context of differences stages of lactation, cow parity, possible breed differences, and the effect of different cattle feeding systems, will require more investigation.

Chapter 5 Variation in *SCD1* and its association with milk traits and milk fatty acid composition

Stearoyl-CoA desaturase 1 (SCD1), also named Δ9-desaturase, can introduce a double-bond at the Δ9,10 position in a large spectrum of FAs, and it is a rate-limiting enzyme in catalysing the synthesis of monounsaturated fatty acids from saturated FAs (Nakamura *et al.*, 2004; Paton *et al.*, 2009). The main substrates for SCD1 are C16:0 and C18:0 FA, which can be converted into C16:1 *cis*-9 and C18:1 *cis*-9 (Figure 5.1). However, SCD1 can also catalyse the addition of a double-bond at the Δ9,10 position in other monounsaturated fatty acyl-CoA substrates. For example, it can catalyse the formation of *cis*-9, *trans*-11 CLA from C18:1 *trans*-11 (Ntambi *et al.*, 2004).

Figure 5. 1 Proposed model of channeling of endogenously synthesised monounsaturated fatty acids from SCD-1 to diacylglycerol acyltransferase (DGAT) during the synthesis of triglycerides (TAG) or acylCoA. Cholesterol acyltrans-ferase (ACAT) in the synthesis of cholesterol esters (CE). Palmitate (16:0) and stearate (18:0) from the diet or de-novo synthesis of fatty acids are desaturated by SCD-1 and channeled to DGAT or ACAT in TG or cholesterol ester synthesis in the ER, respectively (Paton et al., 2009).

The gene for SCD1 (*SCD1*) is located on bovine chromosome 26 and is expressed in a variety of tissues including adipose tissue and mammary gland tissue (Chung *et al.*, 2000). In lactating ruminants, the expression of *SCD1* occurs at high levels (Bernard *et al.*, 2005; M. Bionaz *et al.*, 2008b; McDonald *et al.*, 1973), and the effect of *SCD1* is considered to be important with respect to milk fat composition in these animals (Gautier *et al.*, 2006).

Ntambi *et al.* (2004) reported that dietary factors could regulate the expression of *SCD1*, including the intake of glucose, fructose, vitamin A, cholesterol, vitamin D, polyunsaturated fatty acids, alcohol, conjugated linoleic acid, zinc and cobalt. The expression patterns of *SCD1* in cows that are grazing outdoor on pasture might therefore be to cows fed indoor on supplements. The effect of *SCD1* on milk fat composition in Kiwicross^{TM} cows has not been reported.

Other than the effect of dietary factors, nucleotide sequence variation in *SCD1* is another factor that can change milk FA composition. For example, a nonsynonymous nucleotide substitution in *SCD1* exon 5 (c.878C>T), which causes the substitution of valine with alanine at position 293 of the protein (p.A293V), has been reported by Taniguchi *et al.* (2004) to be associated with carcass FA composition in Japanese Black cattle (Figure 5.2). There was a linkage between the variants c.702A>G, c.762T>C and c.878C>T, with haplotype *A* having the nucleotides A, T and C for the substitutions respectively, and haplotype *V*, having G, C and T.

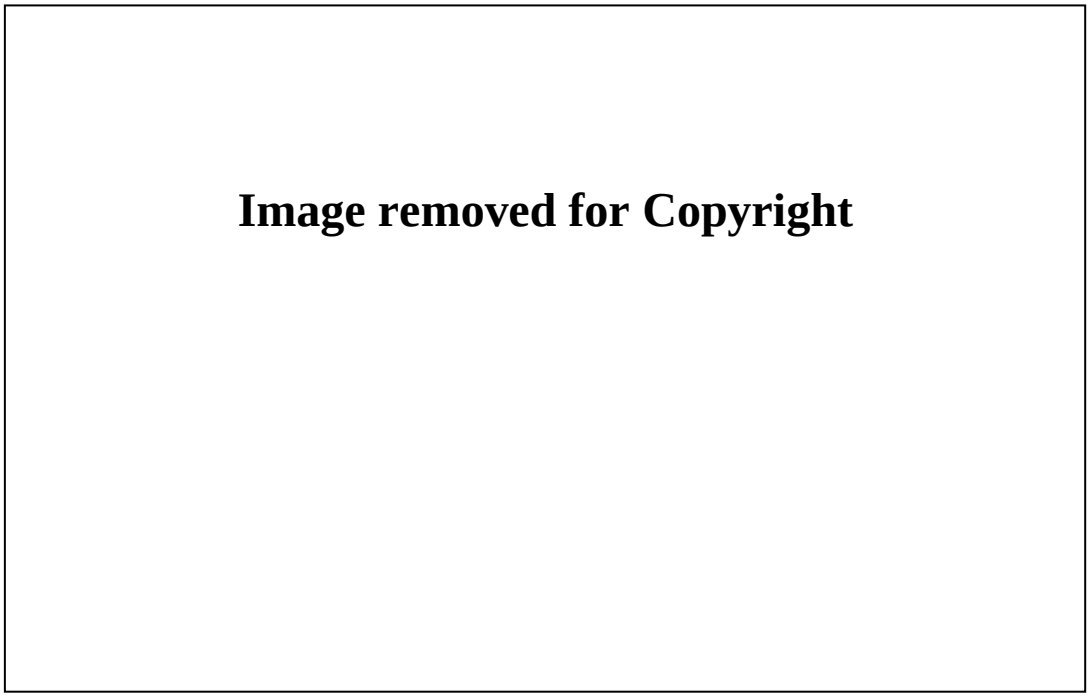


Figure 5. 2 Schematic illustration of full-length bovine SCD cDNA. The positions of eight nucleotide substitutions (triangles above the line) and the associated nucleotide/amino acid changes (boxes). Underlines and hatched boxes show nucleotide substitutions and amino acid replacement, respectively. The large triangle shows the site of amino acid replacement (Taniguchi *et al.*, 2004).

Other studies have not found significant associations between p.A293V and gross milk traits (milk volume, fat percentage and protein percentage), but have associated p.A293V with milk FA composition. For example, Baeza *et al.* (2013) reported that the variants c.702A>G and c.878C>T affected milk MUFA levels and were associated with the C14 and C18 desaturation index. The effects of p.A293V have been studied in different breeds. For example, significant associations were reported between p.A293V and the levels of individual FAs (C10:0 to C18:0) in Italian Holstein, Piedmontese, and Valdostana cattle (Mele *et al.*, 2007; Moioli *et al.*, 2007). A higher frequency of the A allele of p.A293V was found in the Holsteins (0.57) (Mele *et al.*, 2007), Valdostana (0.65) (Moioli *et al.*, 2007), Jerseys (0.94) (Moioli *et al.*, 2007), and Japanese Black cattle (0.59) (Taniguchi *et al.*, 2004) mentioned above. In these breeds, the *SCD1* A allele was always associated with a higher monounsaturated FA content.

Baeza *et al.* (2013) reported that the variant c.*1783A>G (they described this as g.15001A>G) in the 3'UTR, was associated with the C14 desaturation index. In Japanese beef cattle, Taniguchi *et al.* (2004) identified more variants in the 3'UTR of *SCD1*, such as c.*829C>T, c.*2066T/C/G, c.*2273G>A, c.*2458G>A and c.*3649A>T (these are labelled as 1905, 3143, 3351, 3537 and 4736 in Figure 5.2), but the effect of these variations on intra-muscular fat composition were not significant.

There are no reports of the effect of *SCD1* variation on FA traits in Kiwicross^{TM} cows grazed on pasture, thus the effects of *SCD1* on gross milk traits and milk fat composition will be investigated here.

5.1. Materials and methods

5.1.1. Animals and milk sample collection

The Lincoln University Animal Ethics Committee (AEC Number 521) approved this research under the provisions of the Animal Welfare Act 1999 (NZ Government).

The same cows described in the Chapters 3 were investigated here. A total of 450 Holstein-Friesian × Jersey (HF×J) – cross (Kiwicross^{TM}) dairy cows were from two herds (124 cows in herd 1 and 326 cows in herd 2) were investigated. All cows investigated here were 3 to 10 years old and they were grazed on pasture (a mixture of perennial ryegrass and white clover) on the Lincoln University Dairy Farm (LUDF; Canterbury, NZ). All the cows calved over the months August-September, and they were milked twice a day.

Blood samples were collected using the methods described in Chapters 3. The blood samples were analysed at the Lincoln University Gene-Marker Laboratory. A two-step washing procedure (Zhou et al., 2006) was used to purify the genomic DNA.

Samples for milk trait analyses were collected once a month from September 2013 to February 2014. The daily milk yield in litres was recorded using Tru-test milk meters (Tru-test Ltd, Auckland, NZ). These samples were analysed for fat percentage (%) and protein percentage (%) using Fourier-Transform Infra-Red Spectroscopy (MilkoScan FT 120 Foss, Hillerød, Denmark). The milk samples for FA analysis were collected from each cow in a single afternoon milking on 15th January 2014 (days in milk (DIM) = 148 ± 19 days). These were frozen at -20 ^{o}C, and then freeze-dried, prior to being individually ground to a fine powder for component analysis. As in Chapter 3, DNA samples (n = 25) were used to develop a PCR-SSCP protocol for each of the regions of dairy cattle *SCD1*.

5.1.3. Gas Chromatography of the Fatty Acids in the Milk Sample

Gas Chromatography of the fatty acids in the milk samples was described in Chapters 3.

5.1.3. PCR primers used for dairy cattle *SCD1* amplification

Two sets of primers (Table 5.1) were designed to amplify target regions of *SCD1*, based on the cattle reference sequence (NM_173959_4). One region (Region 1) spanned part of intron 4, exon 5 and part of intron 5, and the other (Region 2) spanned part of the 3' UTR (Figure 5.3). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Table 5. 1 Primers used to amplify two regions of the dairy cattle SCD1 gene (*SCD1*).

***SCD1* region[1]**	**Amplicon size (bp)**	**Forward primer**	**Reverse primer**
1	436	5'-AATCAGGTAGGTCTCAGCG-3'	5'-TTCTAATACTGTCCCTTAG-3'
2	397	5'-GAACCACTGTTTCTCTTTAC-3'	5'-CACTTTGGAACCTGCCTTTG-3'

[1] See Figure 5.3

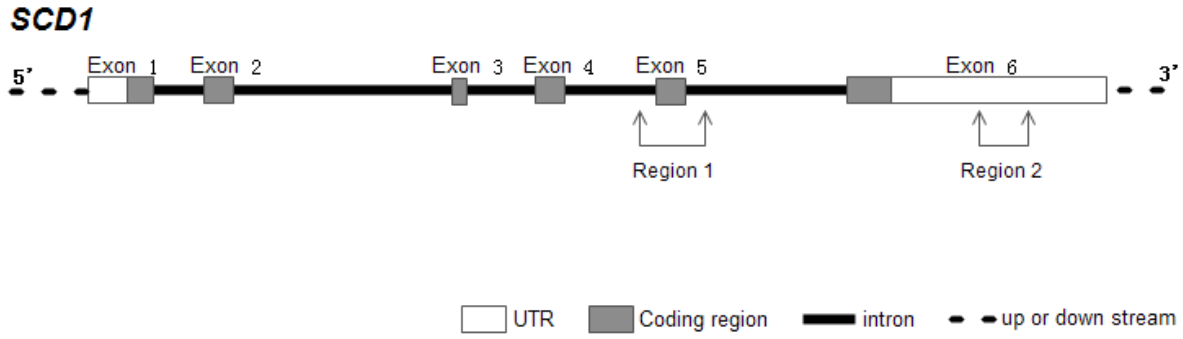


Figure 5. 3 Location of *SCD1* regions that were amplified. Two sets of primers were designed in order to amplify a 436 bp region spanning intron 4 – intron 5 (Region 1), and a 397 bp region in the 3' UTR (Region 2). The gene structure is based on the cattle *SCD1* sequence and is not drawn to scale. Cattle *SCD1* is six-exon long. (Reference sequence number: NM_173959_4).

5.1.4. Developing the PCR-SSCP protocol for *SCD1*

DNA samples (n = 25) were used to develop the PCR-SSCP protocols for analysis of the target regions of dairy cattle *SCD1*. The PCR protocols were optimised with different annealing temperature gradients (between 50 ^{o}C and 62 ^{o}C). Electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels was used to visualise the target amplicons, with 1× TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide.

When the agarose gels produced a satisfactory result, the conditions for band separation and resolution were optimised at different acrylamide percentage gels (10 %, 12 % and 14 %), different concentrations of glycerol (0.5 % - 4 %) and at various temperatures (3 ^{o}C - 35 ^{o}C). For the first test, amplicons were loaded onto 16 cm × 18 cm, 14% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 250 V for 19 hours at 15 ^{o}C in 0.5× TBE buffer.

5.1.5. PCR analysis and genotyping of dairy cattle *SCD1*

Blood samples were collected using the methods described in Chapters 3. The blood samples were analysed at the Lincoln University Gene-Marker Laboratory. A two-step washing procedure (Zhou *et al.*, 2006) was used to purify the genomic DNA.

PCR amplification were performed in a 15-µL reaction containing the purified genomic DNA (a punch of FTA paper), 0.25 µM of each designed primer (Table 5.1), 150 µM of each dNTP (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the polymerase enzyme.

Amplification was undertaken using S1000 thermal cyclers (Bio-Red, Hercules, CA, USA) and the thermal profile included an initial denaturation for two minutes at 94 ^{o}C; followed by 35 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 58 ^{o}C and 30 seconds at 72 ^{o}C; with a final extension for 5 minutes at 72 ^{o}C. Following amplification, a 0.7-µL aliquot of the PCR products was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ^{o}C for 5 minutes and rapid cooling on wet ice, the samples were loaded on 16 cm × 18 cm, acrylamide: bisacrylamide (37.5: 1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) in 0.5× TBE buffer. The method of Byun *et al.* (2009) was used to silver-stain the gels.

For Region 1, the best anneal temperature was 54 ^{o}C. The Region 1 amplicons were loaded onto 16 cm × 18 cm, 12% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels with 2% glycerol, and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 390 V for 19 hours at 12 ^{o}C in 0.5× TBE buffer.

For Region 2, the best anneal temperature was 56 ^{o}C. The Region 2 amplicons were loaded onto 16 cm × 18 cm, 12% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels with 2% glycerol, and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 390 V for 19 hours at 12 ^{o}C in 0.5× TBE buffer.

5.1.6. Sequencing of the dairy cattle *SCD1* Region 1 and 2 variants

Homozygous PCR amplicons identified using PCR-SSCP were sequenced at the Lincoln University DNA Sequencing Facility. If there were not any homozygous samples, single bands of interest from the heterozygous were recovered directly from the SSCP gels as a gel slice. This was macerated and the DNA was eluted into 50 µL TE buffer by incubating at 70 ^{o}C for 20 minutes. The original primers and 1 µL of the eluted solution (as a template) were used for a second round of PCR amplification to produce a simple SSCP gel pattern which could be directly compared to, or found in, the pattern derived from the original heterogeneous amplicons. When banding patterns could be matched and identified, then the

second PCR amplicons were directly sequenced at the Lincoln University DNA Sequencing Facility. The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment and comparisons. The BLAST algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

5.1.7. Statistical analysis

Hardy-Weinberg equilibrium (HWE) for the *SCD1* genotypes was analysed using an online chi-square calculator (http://www.oege.org/software/hwe-mr-calc.shtml).

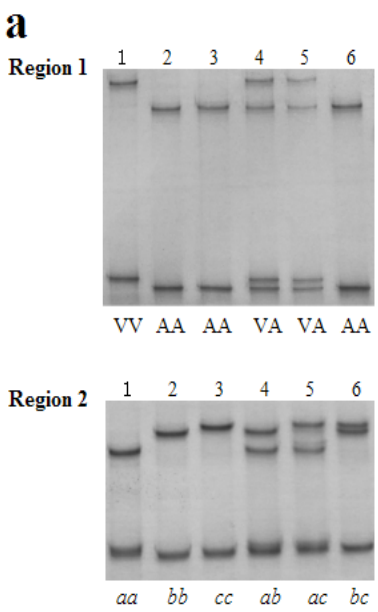
All statistical analysis was carried out using IBM SPSS version 22 (IBM, NY, USA). Associations between variation in *SCD1* and variation in milk FA traits were tested using General Linear Mixed-effects Models (GLMMs). As some measurements were made in percentages, a gamma regression function was adopted in the GLMMs. Single-haplotype presence/absence models (fixed effects: DIM, age and herd) were used to ascertain which haplotypes should be analysed in subsequent multi-haplotype models. The multi-haplotype models included any haplotype that had a haplotype-FA trait association in the single-haplotype presence/absence analysis with a *P* - value of less than 0.200. The multi-haplotype models were again corrected for the fixed effects of (DIM, age and herd) and with haplotype fitted as a random effect. A GLMM (fixed effect: genotype, DIM, age and herd) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes with a frequency greater than 5% (thus insuring adequate sample size), on milk FA traits.

The effect of cow sire could not be included in the GLMMs. Some semen straws (sire genetics) used in NZ dairy cattle artificial insemination based breeding approaches, contain mixed-sire semen purchased from commercial semen producers. In these cases, individual sire identity is impossible to ascertain, but because the straws were mixed-semen straws and because different sires are used for different inseminations in different years, it is unlikely that sire was a strongly confounding effect. Cow age and herd might also be confounded with sire, but this cannot be confirmed.

5.2. Results

5.2.1. Variation in *SCD1*

Variations were identified in the exon 5, intron 5 and 3' UTR regions of *SCD1*, and PCR-SSCP banding patterns and detailed information were list in the Figure 5.4.



b

Nucleotide substitution	Reference	Haplotypes		
		V	*A*	
		a	*b*	*c*
c.702A>G	rs41255691	G	A	A
c.762T>C	rs41255692	C	T	T
c.878C>T	rs41255693	T	C	C
c.880 + 105A>G	rs383175036	G	A	A
c.*1783A>G	rs41255700	G	G	A
c.*1883C>T	rs41255701	T	C	C
c.*1984G>A	rs41255702	A	G	G
c.*2066T/C/G	rs41255703	C	G	T

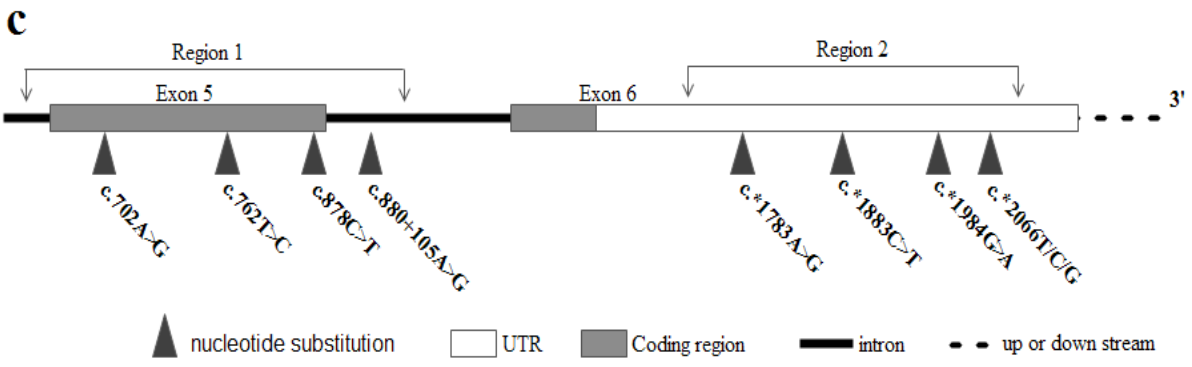


Figure 5. 4 Variation in the bovine SCD1 gene (*SCD1*). (a) Two and three PCR-SSCP banding patterns were identified in Regions 1 and 2 respectively. (b) Two haplotype sequences (corresponding at the nucleotide sequence level to the previously reported V and A alleles) were detected in the intron 4–intron 5 region, and three haplotype sequences (*a*, *b* and *c*) were detected in the 3' UTR region. (c) Of the eight nucleotide substitutions identified, three were located in exon 5, one was in intron 5 and four were in the 3' UTR. The gene structure is based on the cattle *SCD1* sequence (Not drawn to scale, Reference sequence number: NM_173959_4).

5.2.2. Milk traits, milk fat compositions and *SCD1* variation

The associations between *SCD1* variation described here and the gross milk traits (milk yield, milk fat percentage and milk protein percentage) were analysed in this study. However, there were no associations observed (Results are not presented here).

The results for the milk FA trait analyses are described in Chapter 3. Table 5.2 summarise the associations revealed between the composition of milk fat and the *SCD1* genotypes from p.A293V.

Table 5. 2 Association between milk fatty acid levels and p.A293V genotypes

FAME	Mean FAME level ± SE[1] (g/100 g milk FA)			*P*[2]
	VV	AV	AA	
	n = 68	n = 225	n = 149	
C10:0	3.358 ± 0.061[a]	3.231 ± 0.049[b]	3.159 ± 0.054[b]	**0.002**
C10:1	0.256 ± 0.006[c]	0.289 ± 0.005[b]	0.323 ± 0.005[a]	**< 0.001**
C11:0	0.064 ± 0.003[a]	0.057 ± 0.002[b]	0.056 ± 0.003[b]	**0.011**
C13:0 *iso*	0.071 ± 0.001[c]	0.081 ± 0.002[b]	0.091 ± 0.002[a]	**< 0.001**
C12:1	0.086 ± 0.003[c]	0.093 ± 0.002[b]	0.100 ± 0.003[a]	**< 0.001**
C13:0	0.127 ± 0.004[a]	0.117 ± 0.003[b]	0.115 ± 0.004[b]	**0.005**
C14:0	12.919 ± 0.141[a]	12.526 ± 0.112[b]	12.362 ± 0.124[b]	**< 0.001**
C14:1 *cis*-9	0.798 ± 0.027[c]	0.982 ± 0.021[b]	1.165 ± 0.024[a]	**< 0.001**
C16:1	1.448 ± 0.039[a]	1.354 ± 0.031[b]	1.246 ± 0.034[c]	**< 0.001**
C17:0 *iso*	0.542 ± 0.011[b]	0.563 ± 0.008[a]	0.578 ± 0.009[a]	**0.001**
C17:1	0.209 ± 0.004[a]	0.202 ± 0.003[ab]	0.199 ± 0.004[b]	**0.013**
C18:2 *cis*-9, *trans*-13	0.297 ± 0.006[a]	0.286 ± 0.005[ab]	0.280 ± 0.005[b]	**0.004**
C18:2 *cis*-9, *trans*-12	0.080 ± 0.004[a]	0.075 ± 0.003[a]	0.066 ± 0.003[b]	**< 0.001**
MCFA	21.534 ± 0.273[a]	20.875 ± 0.218[b]	20.580 ± 0.240[b]	**0.001**
C10:1 index	7.188 ± 0.197[c]	8.302 ± 0.157[b]	9.370 ± 0.173[a]	**< 0.001**
C12:1 index	2.086 ± 0.051[c]	2.313 ± 0.041[b]	2.526 ± 0.045[a]	**< 0.001**
C14:1 index	5.821 ± 0.194[c]	7.271 ± 0.155[b]	8.638 ± 0.170[a]	**< 0.001**
C16:1 index	3.723 ± 0.086[a]	3.439 ± 0.069[b]	3.180 ± 0.076[c]	**< 0.001**

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted to the models as fixed effects. Means within a row that do not share a superscript letter are separated by Bonferroni test at $P < 0.05$.
[2] $P < 0.05$ in bold.

Table 5.3 and 5.4 summarise the associations revealed between the *SCD1* haplotypes *a*, *b* and *c*, and the composition of individual and grouped FAs respectively. Results were not presented if no association was found. The presence of haplotype *a* was associated with lower C10:1 index, C12:1 index, and C14:1 index values, but elevated C16:1 levels. Haplotypes *b* or *c* appeared to have an opposite effect on the unsaturation of milk FAs, when compared to haplotype *a*. Specifically, there were higher C10:1 index, C12:1 index, C14:1 index and C18:1 index values, when haplotype *b* was present.

Table 5. 3 Association between individual fatty acid levels and *SCD1* 3' UTR variation

FAME	Haplotype	Other haplotype in model	Mean FAME level ± SE[1] (g/100 g milk FA) Absent	n	Present	n	*P*[2]
C10:0	*a*	none	3.156 ± 0.053	157	3.264 ± 0.047	293	**0.004**
	b	none	3.259 ± 0.048	346	3.170 ± 0.055	104	**0.035**
	c	none	3.280 ± 0.053	124	3.209 ± 0.049	326	*0.076*
	a	*b, c*	3.163 ± 0.068	157	3.249 ± 0.063	293	**0.030**
	b	*a, c*	3.260 ± 0.069	346	3.161 ± 0.073	104	**0.029**
	c	*a, b*	3.256 ± 0.079	124	3.175 ± 0.075	326	*0.067*
C10:1	*a*	none	0.324 ± 0.006	157	0.281 ± 0.005	293	**< 0.001**
	b	none	0.284 ± 0.005	346	0.314 ± 0.006	104	**< 0.001**
	c	none	0.277 ± 0.006	124	0.301 ± 0.006	326	**< 0.001**
	a	*b, c*	0.319 ± 0.018	157	0.287 ± 0.018	293	**< 0.001**
	b	*a, c*	0.289 ± 0.019	346	0.318 ± 0.019	104	**< 0.001**
	c	*a, b*	0.292 ± 0.022	124	0.313 ± 0.022	326	**< 0.001**
C13:0 *iso*	*a*	none	0.092 ± 0.002	157	0.079 ± 0.002	293	**< 0.001**
	b	none	0.080 ± 0.002	346	0.090 ± 0.002	104	**< 0.001**
	c	none	0.078 ± 0.002	124	0.085 ± 0.002	326	**< 0.001**
	a	*b, c*	0.091 ± 0.006	157	0.081 ± 0.006	293	**< 0.001**
	b	*a, c*	0.081 ± 0.006	346	0.091 ± 0.006	104	**< 0.001**
	c	*a, b*	0.083 ± 0.007	124	0.089 ± 0.007	326	**0.002**
C12:1	*a*	none	0.101 ± 0.003	157	0.091 ± 0.002	293	**< 0.001**
	b	none	0.092 ± 0.002	346	0.100 ± 0.003	104	**< 0.001**
	c	none	0.092 ± 0.003	124	0.095 ± 0.002	326	*0.074*
	a	*b, c*	0.101 ± 0.005	157	0.093 ± 0.004	293	**< 0.001**
	b	*a, c*	0.093 ± 0.005	346	0.101 ± 0.005	104	**< 0.001**
	c	*a, b*	0.095 ± 0.006	124	0.099 ± 0.006	326	*0.126*
C14:0	*a*	none	12.359 ± 0.124	157	12.629 ± 0.109	293	**0.002**
	b	none	12.653 ± 0.109	346	12.301 ± 0.127	104	**< 0.001**
	c	none	12.652 ± 0.123	124	12.503 ± 0.113	326	*0.109*
	a	*b, c*	12.387 ± 0.235	157	12.542 ± 0.228	293	*0.101*
	b	*a, c*	12.680 ± 0.169	346	12.275 ± 0.178	104	**< 0.001**
	c	*a, b*	12.610 ± 0.244	124	12.363 ± 0.240	326	**0.015**
C14:1	*a*	none	1.173 ± 0.026	157	0.934 ± 0.023	293	**< 0.001**
	b	none	0.957 ± 0.026	346	1.106 ± 0.030	104	**< 0.001**
	c	none	0.912 ± 0.029	124	1.047 ± 0.027	326	**< 0.001**
	a	*b, c*	1.144 ± 0.092	157	0.967 ± 0.091	293	**< 0.001**
	b	*a, c*	0.984 ± 0.106	346	1.127 ± 0.107	104	**< 0.001**
	c	*a, b*	0.999 ± 0.116	124	1.111 ± 0.115	326	**< 0.001**
C16:1	*a*	none	1.265 ± 0.035	157	1.373 ± 0.031	293	**< 0.001**
	b	none	1.330 ± 0.032	346	1.384 ± 0.037	104	*0.057*
	c	none	1.438± 0.034	124	1.291 ± 0.031	326	**< 0.001**
	a	*b, c*	1.307 ± 0.067	157	1.384 ± 0.065	293	**0.004**
	b	*a, c*	1.330 ± 0.074	346	1.369 ± 0.075	104	0.203
	c	*a, b*	1.410 ± 0.049	124	1.287 ± 0.046	326	**< 0.001**
C17:0 *iso*	*a*	none	0.580 ± 0.009	157	0.558 ± 0.008	293	**0.001**
	b	none	0.559 ± 0.008	346	0.576 ± 0.010	104	**0.017**
	c	none	0.557 ± 0.009	124	0.567 ± 0.009	326	*0.158*
	a	*b, c*	0.581 ± 0.011	157	0.560 ± 0.010	293	**0.002**
	b	*a, c*	0.563 ± 0.013	346	0.578 ± 0.013	104	*0.056*
	c	*a, b*	0.565 ± 0.015	124	0.573 ± 0.014	326	0.278
C17:1	*a*	none	0.200 ± 0.004	157	0.204 ± 0.003	293	**0.023**
	b	none	0.201 ± 0.003	346	0.207 ± 0.004	104	**0.024**
	c	none	0.209 ± 0.004	124	0.199 ± 0.003	326	**< 0.001**
	a	*b, c*	0.203 ± 0.006	157	0.205 ± 0.005	293	0.473
	b	*a, c*	0.203 ± 0.005	346	0.207 ± 0.005	104	*0.188*
	c	*a, b*	0.209 ± 0.004	124	0.200 ± 0.003	326	**0.001**
C18:1 *cis*-9	*a*	none	12.269 ± 0.222	157	13.049 ± 0.196	293	*0.154*
	b	none	12.968 ± 0.196	346	13.482 ± 0.228	104	**0.003**
	c	none	13.152 ± 0.219	124	13.083 ± 0.201	326	0.675
	a	b	13.306 ± 0.315	157	13.165 ± 0.301	293	0.369
	b	a	12.968 ± 0.196	346	13.482 ± 0.228	104	**0.003**
C18:2 *trans*-9, 12	*a*	none	0.405 ± 0.006	157	0.397 ± 0.005	293	**0.042**

	b	none	0.401 ± 0.005	346	0.396 ± 0.006	104	0.268
	c	none	0.393 ± 0.006	124	0.403 ± 0.005	326	**0.024**
	a	*c*	0.404 ± 0.007	157	0.397 ± 0.006	293	*0.117*
	c	*a*	0.394 ± 0.006	124	0.403 ± 0.006	326	**0.046**
C18:2 *cis*-9, *trans*-13	*a*	none	0.281 ± 0.005	157	0.289 ± 0.005	293	**0.021**
	b	none	0.286 ± 0.005	346	0.287 ± 0.005	104	0.804
	c	none	0.293 ± 0.005	124	0.283 ± 0.005	326	**0.015**
	a	*c*	0.282 ± 0.006	157	0.289 ± 0.005	293	*0.073*
	c	*a*	0.291 ± 0.006	124	0.283 ± 0.005	326	**0.045**
C18:2 *cis*-9, *trans*-12	*a*	none	0.066 ± 0.003	157	0.076 ± 0.003	293	**< 0.001**
	b	none	0.073 ± 0.003	346	0.074 ± 0.003	104	0.768
	c	none	0.079 ± 0.003	124	0.070 ± 0.003	326	**< 0.001**
	a	*c*	0.068 ± 0.004	157	0.076 ± 0.004	293	**0.001**
	c	*a*	0.076 ± 0.005	124	0.070 ± 0.005	326	**0.018**
C19:0	*a*	none	0.136 ± 0.004	157	0.131 ± 0.003	293	**0.036**
	b	none	0.133 ± 0.003	346	0.130 ± 0.004	104	0.217
	c	none	0.128 ± 0.004	124	0.135 ± 0.003	326	**0.026**
	a	*c*	0.135 ± 0.004	157	0.131 ± 0.004	293	*0.099*
	c	*a*	0.129 ± 0.004	124	0.135 ± 0.004	326	*0.055*
C20:0	*a*	none	0.119 ± 0.002	157	0.123 ± 0.002	293	**0.011**
	b	none	0.123 ± 0.002	346	0.118 ± 0.003	104	**0.011**
	c	none	0.123 ± 0.002	124	0.121 ± 0.002	326	0.380
	a	*b*	0.119 ± 0.003	157	0.122 ± 0.003	293	**0.031**
	b	*a*	0.122 ± 0.003	346	0.118 ± 0.003	104	**0.029**
C22.5 *cis*-7, 10, 13, 16, 19	*a*	none	0.124 ± 0.004	157	0.119 ± 0.003	293	**0.041**
	b	none	0.119 ± 0.003	346	0.122 ± 0.004	104	0.387
	c	none	0.121 ± 0.004	124	0.120 ± 0.003	326	0.687

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted to the models as fixed effects.
[2] $0.05 < P < 0.2$ in italics; $P < 0.05$ in bold.

Table 5. 4 Association between grouped fatty acid levels and *SCD1* 3' UTR variation

FAME	Haplotype	Other haplotype in model	Mean FAME level ± SE[1] (g/100 g milk FA) Absent	n	Present	n	*P*[2]
MCFA	*a*	none	20.574 ± 0.239	157	21.047 ± 0.211	293	**0.005**
	b	none	21.062 ± 0.213	346	20.541 ± 0.247	104	**0.006**
	c	none	21.112 ± 0.237	124	20.810 ± 0.217	326	*0.093*
	a	*b, c*	20.622 ± 0.378	157	20.930 ± 0.359	293	*0.090*
	b	*a, c*	21.106 ± 0.314	346	20.495 ± 0.333	104	**0.003**
	c	*a, b*	21.040 ± 0.400	124	20.603 ± 0.390	326	**0.026**
Total C18:1	*a*	none	16.722 ± 0.257	157	16.464 ± 0.226	293	*0.148*
	b	none	16.412 ± 0.227	346	16.858 ± 0.264	104	**0.027**
	c	none	16.546 ± 0.253	124	16.526 ± 0.232	326	0.920
	a	*b*	16.748 ± 0.309	157	16.547 ± 0.288	293	0.266
	b	*a*	16.412 ± 0.227	346	16.857 ± 0.264	104	**0.027**
MUFA	*a*	none	20.409 ± 0.264	157	19.975 ± 0.233	293	**0.018**
	b	none	19.902 ± 0.233	346	20.596 ± 0.271	104	**0.001**
	c	none	20.100 ± 0.261	124	20.087 ± 0.240	326	0.951
	a	*b*	20.457 ± 0.390	157	20.127 ± 0.374	293	*0.075*
	b	*a*	19.964 ± 0.269	346	20.607 ± 0.299	104	**0.002**
Total branched FA	*a*	none	1.633 ± 0.022	157	1.598 ± 0.019	293	**0.018**
	b	none	1.602 ± 0.019	346	1.620 ± 0.022	104	0.300
	c	none	1.594 ± 0.021	124	1.615 ± 0.020	326	0.212
Total UFA	*a*	none	24.453 ± 0.314	157	24.029 ± 0.277	293	*0.052*
	b	none	23.958 ± 0.277	346	24.637 ± 0.323	104	**0.006**
	c	none	24.151 ± 0.310	124	24.138 ± 0.285	326	0.955
	a	*b*	24.497 ± 0.416	157	24.168 ± 0.394	293	*0.137*
	b	*a*	24.002 ± 0.301	346	24.645 ± 0.340	104	**0.010**
Total SFA	*a*	none	68.462 ± 0.342	157	68.861 ± 0.301	293	*0.093*
	b	none	68.925 ± 0.303	346	68.299 ± 0.352	104	**0.020**
	c	none	68.724 ± 0.337	124	68.772 ± 0.310	326	0.850

	a	*b*	68.426 ± 0.418	157	68.745 ± 0.391	293	*0.185*
	b	*a*	68.897 ± 0.316	346	68.294 ± 0.362	104	**0.026**
Total index	*a*	none	26.323 ± 0.342	157	28.873 ± 0.301	293	*0.058*
	b	none	25.799 ± 0.302	346	26.513 ± 0.352	104	**0.008**
	c	none	26.009 ± 0.338	124	25.985 ± 0.310	326	0.925
	a	*b*	26.368 ± 0.445	157	26.017 ± 0.420	293	*0.145*
	b	*a*	25.844 ± 0.326	346	26.521 ± 0.369	104	**0.012**
MUFA index	*a*	none	23.093 ± 0.315	157	22.601 ± 0.278	293	**0.025**
	b	none	22.524 ± 0.278	346	23.290 ± 0.324	104	**0.002**
	c	none	22.740 ± 0.312	124	22.729 ± 0.286	326	0.965
	a	*b*	23.144 ± 0.444	157	22.763 ± 0.423	293	*0.086*
	b	*a*	22.591 ± 0.317	346	23.302 ± 0.354	104	**0.005**
C10:1 index	*a*	none	9.408 ± 0.184	157	8.012 ± 0.162	293	**< 0.001**
	b	none	8.120 ± 0.177	346	9.096 ± 0.206	104	**< 0.001**
	c	none	7.899 ± 0.198	124	8.664 ± 0.182	326	**< 0.001**
	a	*b, c*	9.250 ± 0.595	157	8.237 ± 0.589	293	**< 0.001**
	b	*a, c*	8.266 ± 0.623	346	9.217 ± 0.626	104	**< 0.001**
	c	*a, b*	8.396 ± 0.711	124	9.072 ± 0.706	326	**< 0.001**
C12:1 index	*a*	none	2.543 ± 0.048	157	2.252 ± 0.042	293	**< 0.001**
	b	none	2.262 ± 0.044	346	2.511 ± 0.051	104	**< 0.001**
	c	none	2.252 ± 0.050	124	2.375 ± 0.046	326	**0.001**
	a	*b, c*	2.525 ± 0.134	157	2.310 ± 0.132	293	**< 0.001**
	b	*a, c*	2.300 ± 0.127	346	2.533 ± 0.128	104	**< 0.001**
	c	*a, b*	2.356 ± 0.164	124	2.472 ± 0.162	326	**0.004**
C14:1 index	*a*	none	8.692 ± 0.190	157	6.893 ± 0.168	293	**< 0.001**
	b	none	7.047 ± 0.191	346	8.252 ± 0.222	104	**< 0.001**
	c	none	6.728 ± 0.214	124	7.745 ± 0.197	326	**< 0.001**
	a	*b, c*	8.472 ± 0.748	157	7.173 ± 0.742	293	**< 0.001**
	b	*a, c*	7.230 ± 0.797	346	8.411 ± 0.0.800	104	**< 0.001**
	c	*a, b*	7.372 ± 0.891	124	8.258 ± 0.887	326	**< 0.001**
C16:1 index	*a*	none	3.227 ± 0.078	157	3.500 ± 0.069	293	**< 0.001**
	b	none	3.393 ± 0.071	346	3.518 ± 0.083	104	**0.047**
	c	none	3.671 ± 0.076	124	3.288 ± 0.069	326	**< 0.001**
	a	*b, c*	3.340 ± 0.172	157	3.523 ± 0.167	293	**0.002**
	b	*a, c*	3.402 ± 0.187	346	3.479 ± 0.190	104	0.251
	c	*a, b*	3.600 ± 0.112	124	3.274 ± 0.106	326	**< 0.001**
C18:1 index	*a*	none	61.234 ± 0.515	157	61.016 ± 0.454	293	0.542
	b	none	60.747 ± 0.453	346	61.946 ± 0.527	104	**0.003**
	c	none	61.365 ± 0.506	124	60.909 ± 0.464	326	0.234
CLA index	*a*	none	27.229 ± 0.420	157	27.557 ± 0.370	293	0.259
	b	none	27.253 ± 0.371	346	28.045 ± 0.432	104	**0.016**
	c	none	27.954 ± 0.411	124	27.193 ± 0.377	326	**0.015**
	b	*c*	27.344 ± 0.446	346	28.003 ± 0.494	104	*0.053*
	c	*b*	27.968 ± 0.477	124	27.327 ± 0.458	326	**0.049**

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted to the models as fixed effects.
[2] $0.05 < P < 0.2$ in italics; $P < 0.05$ in bold.

Table 5.5 summarises the associations revealed between the composition of milk fat and the *SCD1* genotypes from the 3' UTR variant. Results are only presented if an association is found. The frequency of *bb* genotype was too low (1.8%), thus, the animals with *bb* type were not included in the analyses. With this analysis, the effects of the p.A293V amino acid substitution and the variants in 3'UTR of *SCD1* on most milk fat compositions were similar. The effects of c.*2066T/C/G and c.*1783A>G on milk fat composition could be observed as the difference between *bc* and *cc* genotype cows. There was less C16:1, C17:1, C18:2 *cis*-9, *trans*-13 and C18:2 *cis*-9, *trans*-12 in the milk from *cc* cows.

Table 5. 5 Association between milk fatty acid levels and 3' UTR genotypes[1].

FAME	Mean FAME level ± SE[2] (g/100 g milk FA)					P[3]
	aa	***ab***	***ac***	***bc***	***cc***	
	n = 68	n = 48	n = 177	n = 48	n = 101	
C10:0	3.361 ± 0.061^{a}	3.202 ± 0.066^{ab}	3.242 ± 0.051^{ab}	3.136 ± 0.069^{b}	3.174 ± 0.058^{b}	**0.009**
C10:1	0.255 ± 0.006^{c}	0.297 ± 0.006^{b}	0.286 ± 0.005^{b}	0.332 ± 0.007^{a}	0.317 ± 0.006^{a}	**< 0.001**
C11:0	0.064 ± 0.003	0.056 ± 0.003	0.058 ± 0.002	0.056 ± 0.003	0.056 ± 0.003	**0.047**
C13:0 *iso*	0.071 ± 0.002^{d}	0.085 ± 0.003^{bc}	0.080 ± 0.002^{c}	0.095 ± 0.003^{a}	0.089 ± 0.002^{ab}	**< 0.001**
C12:1	0.086 ± 0.003^{c}	0.097 ± 0.003^{ab}	0.092 ± 0.002^{bc}	0.103 ± 0.003^{a}	0.098 ± 0.003^{a}	**< 0.001**
C13:0	0.127 ± 0.004^{a}	0.113 ± 0.005^{b}	0.118 ± 0.004^{ab}	0.115 ± 0.005^{ab}	0.115 ± 0.004^{b}	**0.018**
C14:0	12.933 ± 0.140^{a}	12.352 ± 0.151^{b}	12.594 ± 0.118^{ab}	12.231 ± 0.158^{b}	12.448 ± 0.134^{b}	**< 0.001**
C14:1 *cis*-9	0.796 ± 0.027^{c}	1.010 ± 0.029^{b}	0.970 ± 0.023^{b}	1.204 ± 0.030^{a}	1.141 ± 0.025^{a}	**< 0.001**
C16:1	1.444 ± 0.039^{a}	1.408 ± 0.042^{ab}	1.332 ± 0.033^{b}	1.300 ± 0.044^{bc}	1.213 ± 0.037^{c}	**< 0.001**
C17:0 *iso*	0.541 ± 0.011^{b}	0.570 ± 0.011^{ab}	0.561 ± 0.009^{ab}	0.580 ± 0.012^{a}	0.577 ± 0.010^{a}	**0.004**
C17:1	0.209 ± 0.004^{a}	0.207 ± 0.004^{ab}	0.201 ± 0.003^{ab}	0.204 ± 0.005^{ab}	0.195 ± 0.004^{b}	**0.004**
C18:2 *cis*-9, *trans*-13	0.297 ± 0.006^{a}	0.286 ± 0.006^{ab}	0.286 ± 0.005^{ab}	0.286 ± 0.007^{ab}	0.277 ± 0.006^{b}	**0.011**
C18:2 *cis*-9, *trans*-12	0.080 ± 0.004^{a}	0.078 ± 0.004^{a}	0.073 ± 0.003^{a}	0.068 ± 0.004^{ab}	0.065 ± 0.004^{b}	**< 0.001**
C20:0	0.125 ± 0.003^{a}	0.122 ± 0.003^{ab}	0.123 ± 0.002^{ab}	0.116 ± 0.003^{b}	0.121 ± 0.003^{ab}	**0.044**
MCFA	21.552 ± 0.273^{a}	20.655 ± 0.295^{b}	20.962 ± 0.230^{ab}	20.400 ± 0.309^{b}	20.698 ± 0.260^{b}	**0.002**
MUFA	19.866 ± 0.300^{b}	20.244 ± 0.323^{ab}	19.924 ± 0.252^{b}	20.883 ± 0.339^{a}	20.064 ± 0.285^{ab}	**0.020**
MUFA index	22.502 ± 0.359^{ab}	22.865 ± 0.387^{ab}	22.546 ± 0.302^{b}	23.637 ± 0.405^{a}	22.698 ± 0.341^{ab}	**0.036**
C10:1 index	7.166 ± 0.196^{c}	8.558 ± 0.211^{b}	8.198 ± 0.165^{b}	9.671 ± 0.221^{a}	9.187 ± 0.186^{a}	**< 0.001**
C12:1 index	2.079 ± 0.051^{d}	2.395 ± 0.055^{bc}	2.280 ± 0.043^{c}	2.611 ± 0.057^{a}	2.473 ± 0.048^{ab}	**< 0.001**
C14:1 index	5.797 ± 0.192^{c}	7.542 ± 0.207^{b}	7.160 ± 0.161^{b}	8.992 ± 0.216^{a}	8.426 ± 0.182^{a}	**< 0.001**
C16:1 index	3.712 ± 0.085^{a}	3.568 ± 0.092^{ab}	3.387 ± 0.072^{b}	3.326 ± 0.096^{bc}	3.090 ± 0.081^{c}	**< 0.001**
CLA index	27.789 ± 0.470	27.818 ± 0.507	27.376 ± 0.395	27.799 ± 0.530	26.629 ± 0.447	**0.037**

[1] The genotypes with a frequency greater than5% were analysed. The frequency of bb (n = 8) is 1.78 %. The insignificant results were not show in the table.
[2] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted to the models as fixed effects. Means within a row that do not share a superscript letter are separated by Bonferroni test at $P < 0.05$.
[3] $P < 0.05$ in bold.

5.3. Discussion

The enzyme SCD1 is a rate-limiting enzyme in the biosynthesis of many monounsaturated FAs (Ntambi *et al.*, 2004), and sequence variation in SCD1 has been revealed to affect milk fat composition. The SCD1 gene is therefore considered to be a useful candidate gene for use in breeding programmes targeted at improving the nutritional value of milk. The most commonly described variation in *SCD1* is the variant c.878C>T located in exon 5. It underpins the substitution of the amino acid valine (V) with alanine (A) at amino acid position 293 in the SCD1 protein. The function of SCD1 is likely to be affected by p.A293V because it is located in the third histidine-rich region of the enzyme. This histidine-rich region has been revealed to be important for the catalytic activity of the enzyme (Shanklin *et al.*, 1994).

There was linkage between c.878C>T and other variants. Taniguchi *et al.* (2004) identified linkage between three nucleotide substitutions c.702A>G, c.762T>C and c.878C>T in exon 5 of Japanese black cattle. Based on these three variants, they described the haplotype p.293V (GCT) and p.293A (ATC). This linkage was also found by Baeza *et al.* (2013) with the variant g.10153A>G (equal to c.702A>G here) being in complete linkage disequilibrium with c.878C>T in the beef cattle they studied. In this study, three nucleotide variations in exon 5 (c.702A>G, c.762T>C and c.878C>T), one variant in intron 5 (c.880+105A>G) and four variants in the 3'UTR (c.*1883C>T, c.*1984G>A, c.*1783A>G and c.*2066T/C/G) were revealed. There was linkage between c.702A>G, c.762T>C, c.878C>T, c.880 + 105A>G, c.*1883C>T and c.*1984G>A (Figure 5.4).

In an *in-vitro* study, Enoch *et al.* (1976) revealed that the acyl-CoA derivatives with 12 to 19 carbon atoms were required as substrates for SCD1 enzyme activity. Schennink *et al.* (2008), Kgwatalala *et al.* (2009b) and this research (Table 5.2) all suggested that *SCD1* p.A293V has effects on individual FAs and index levels, especially on the level of C10:1, C12:1, C14:1, and the C10 index, C12 index, C14 index and C16 index levels. In addition, Enoch *et al.* (1976) found that the SCD1 enzyme has substrate specificity, with a preference for longer-chain FAs. However, the effect of p.A293V on C18 FA levels (such as the C18:1 *trans*-11, C18:1 *cis*- 9, C18 index) were not confirmed.

The previous studies that revealed an effect of p.A293V on C18 FA levels could be influenced by different factors, such as season, genes, breed and the stage of lactation. Duchemin *et al.* (2013) revealed that the p.293V allele was negatively associated with C18:1 *trans*-11 and that this negative effect was larger in summer than in winter. Schennink *et al.*

(2008) investigated the joint effect of *SCD1* and *DGAT1* variation and suggested that the genetic variation explained by *DGAT1* (p.232K) and the genetic variation explained by *SCD1* (p.293A) are additive with respect to their effect on C16, C18 and CLA levels. Moioli *et al.* (2007) revealed that p.A293V affected C10:1, C14:1, C16:1, C10 index and C14 index level significantly in Piedmontese (n = 81), Jersey (n = 75) and Valdostana (n = 730) cows, but the effect of p.293A on C18 level could only be observed when the variant frequency was high (i.e. when at a frequency of 0.94 in Jersey cattle and 0.65 in Valdostana cattle). With a lower frequency of p.293A (i.e. 0.42) in Piedmontese, the effect of *SCD1* variation on C18 levels was not significant. Mele *et al.* (2007) investigated the effect of p.293A and the effect of lactation stage on C18:1 *cis*-9 FA levels. A negative effect was found when the DIM increased ($P < 0.01$), and that p.293A (the frequency was 0.57) had a positive effect on the C18:1 *cis*-9 FA level ($P < 0.05$). In this study, a high frequency of the p.293A allele (59.9 %) was observed in the Kiwicross^{TM} cows. This finding is consistent with the findings of Schennink *et al.* (2008), Kgwatalala *et al.* (2009b) and Mele *et al.* (2007) who reported a similar result, with frequencies of 73 % in Dutch HF cows, 69 % in Canadian HF cows and 57 % in Italian HF cows respectively.

The 3' UTR plays a critical role in translation termination and post-transcriptional gene expression (Barrett *et al.*, 2012). Baeza *et al.* (2013) reported that the 3'UTR variant g.15001A>G (equal to c.*1783A>G here) affected meat fat composition (C14:1 level, $P < 0.05$) in their Argentinian Brangus beef cattle. In the results reported here, after differentiating the p.293A haplotype to *b* and *c* based on 3'UTR typing, their different effects on long chain FA levels were revealed. The decline in C16:1, C17:1, C18:2 *cis*-9, *trans*-13, and C18:2 *cis*-9, *trans*-12 FA levels and C16:1 index level was associated with the presence of haplotype *c*. The presence of haplotype *b* was associated with an increase in C18:1 cis-9 and total C18:1 FA levels, MUFA levels, C18:1 index level and MUFA index level. At the level of genotype (Table 5.5), there was a significant difference between *aa* and *cc* cows in C16:1, C17:1, C18:2 *cis*-9, *trans*-13 and C18:2 *cis*-9, *trans*-12 FA levels. In addition, there was a significant difference between *aa* and *bb* cows for C20:0 and MUFA levels. This suggests the 3'UTR, specifically the substitutions c.*1783A>G and c.*2066T/C/G are better able to resolve the effect of *SCD1* on milk FA levels. In this respect, Kgwatalala *et al.* (2009b) regarded the effect of different lactation stages on milk MUFA was more important than p.A293V, and this along with the results presented here suggests more research into *SCD1* variation and lactation stage is needed to gain clarity into what is driving variation in FA levels.

The influence of 3'UTR variation was also described by Kgwatalala *et al.* (2009a) in 46 Holstein and 35 Jersey cows in Canada. In their study, three haplotypes H1, H2 and H3 (equal

to the haplotypes *c*, *b* and *a* here) were identified with the frequency 67.1%, 2.3% and 30.6% respectively. Significant differences were only found in two milk FA levels, with their H1H1 cows producing more C10:1 and C12:1 than the H3H3 cows (similar results could be found between *cc* and *aa* cows here). Moreover, Kgwatalala *et al.* (2009a) reported that an internal ribosome entry site (IRES) could be found in the *c* variant only. The presence of an IRES motif may ultimately affect SCD1 protein turnover or the quantity of the SCD1 enzyme produced, because it could enhance translation of the constituent mRNA. They suggested that the nucleotide variation in 3'UTR region might lead to the absence of the IRES in the *a* and *b* haplotypes.

With a low frequency of *b* in their Holstein (0.054) and Jersey (0.000) cattle, Kgwatalala *et al.* (2009a) suggested that milk fat composition was not affected by haplotype *b* significantly. Therefore, the variation in C10:1 and C12:1 FA levels in their study could be due to either the variation in the 3'UTR (c.*1783A>G) or the variation in exon 5 (c.878C>T). If this was true, then the effect of *c* on milk fat composition should be the opposite of the effects of *a*, *b* or *a* + *b*. In the Kiwicross^{TM} cows studied here the frequency of *b* was 0.124, and opposite effects were observed between haplotype *c* and the other two haplotypes for C16:1, C18:1 *cis*-9, C18:2 *trans*-9, 12, C18:2 *cis*-9, *trans*-12, C18:2 *cis*-9, *trans*-13, C19:0 FA levels, and C16:1 index levels (Table 5.3 and Table 5.4). At the genotype level (Table 5.5), the *cc* cows produced more C10:1 and C12:1 FA than the *aa* cows. This is similar to what was reported by Kgwatalala *et al.* (2009a). Moreover, the *ab* cows produced less C10:1 and C14:1 *cis*-9 FA, but more C18:2 *cis*-9, *trans*-12 and C16:1 FA, than the *cc* cows.

The effect of *SCD1* on gross milk traits (milk yield, fat and protein percentage) is still in dispute. Macciotta *et al.* (2008) investigated 313 Italian Holstein cows and found that their p.A293V VV cows had higher milk yields and protein yields than their AV and AA cows. The associations they found appear to be consistent across different stages of lactation. However, Mao *et al.* (2012) and Signorelli *et al.* (2009) reported a significant negative effect of the V allele on milk yield in Chinese Holstein, Piedmontese and Valdostana breeds. In addition, Schennink *et al.* (2008) didn't found any significant associations between p.A293V and milk traits in Dutch Holstein, a result that was consistent with our findings here in and Kiwicross^{TM} cows.

Chapter 6 Variation in *PLIN2* and its association with milk traits and milk fat composition

The perilipin-2 gene (*PLIN2* also known as *ADFP*) encodes the protein perilipin-2 (also called the adipose differentiation-related protein (ADRP), and adipophilin). This protein participates in the regulation of body fat distribution and is located on the surface of lipid droplets in different tissues. During lipid droplet formation, upregulation of *PLIN2* expression occurs, along with an increase in lipid storage (Listenberger *et al.*, 2007; Prats *et al.*, 2006).

The *PLIN2* gene is an important candidate gene for fat deposition traits because muscle tissues will uptake more FA for triglyceride formation when abundant *PLIN2* expression occurs (Imai *et al.*, 2007; Imamura *et al.*, 2002; Magra *et al.*, 2006). In cattle, *PLIN2* is located on chromosome 8 (Figure 6.1). Previous investigations have described 25 nucleotide sequence variations in beef cattle and that these variations occur in different gene regions (the promoter region, the coding exons, the untranslated regions and the introns). The location of, and information about these nucleotide sequence variations is summarised in Figure 6.2. Nucleotide sequence variation in *PLIN2* has been identified and associated with intramuscular fat content in chicken (Zhao *et al.*, 2009) and carcass traits in beef cattle (Cheong *et al.*, 2009). In Cheong *et als*' study, the substitution c.-74A>G in the promoter region (equivalent to c.-56-18A>G in Figure 6.2; rs382435864) was associated with meat marbling score.

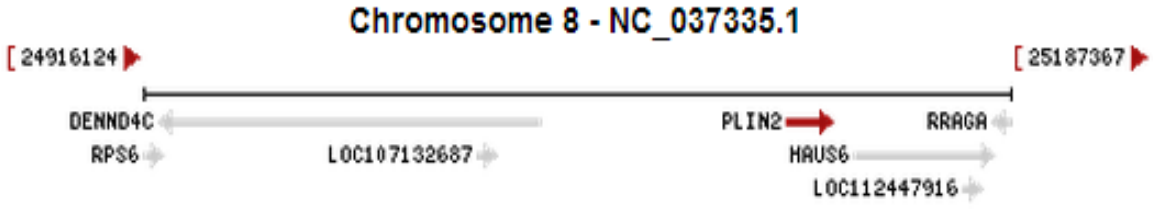


Figure 6. 1 Chromosomal Location of bovine *PLIN2*.

Figure 6. 2 Exon-intron map of *PLIN2* on chromosome 8 in beef cattle (Cheong *et al.*, 2009).

During lactation in dairy cattle, perilipin-2 participates in globule surface membrane formation and it is one of the constituents of the globule surface (Figure 2.8) (McManaman *et al.*, 2007; Reinhardt *et al.*, 2006). Bionaz *et al.* (2008b) have described how the expression of *PLIN2* increases during early lactation (with a peak in expression at the 60th day in milk), then declined subsequently. Li *et al.* (2014) identified seven nucleotide substitutions and six haplotypes of *PLIN2* that were associated with goat milk yield traits. Although *PLIN2* is an ubiquitously expressed gene (Brasaemle *et al.*, 1997), to date there have been no reports of genetic association studies between *PLIN2* variation and milk traits in dairy cattle. Accordingly in this study, variation in *PLIN2* will be searched for in dairy cattle, and if it is identified then associations between that variation and variation in milk traits (milk yield, fat percentage, protein percentage and fat composition) will be investigated.

6.1. Materials and methods

6.1.1. Animals and milk sample collection

The Lincoln University Animal Ethics Committee (AEC Number 521) approved this research under the provisions of the Animal Welfare Act 1999 (NZ Government).

The same cows described in the Chapters 3 were investigated here. A total of 450 Holstein-Friesian × Jersey (HF×J) - cross dairy cows were from two herds (124 cows in herd 1, 326 cows in herd 2) were studied. All the cows investigated were 3 to 10 years old and they were grazed on pasture (a mixture of perennial ryegrass and white clover) on the Lincoln University Dairy Farm (LUDF; Canterbury, NZ). All the cows calved over the months August-September. They were milked twice a day throughout lactation (from calving until the end of May).

Blood samples were collected using the methods described in Chapters 3. The blood samples were analysed at the Lincoln University Gene-Marker Laboratory. A two-step washing procedure (Zhou et al., 2006) was used to purify the genomic DNA.

A milk sample was collected from each cow, in a single afternoon milking on 15th January 2014 (days in milk (DIM) = 148 ± 19 days). The milk samples were frozen at -20 ^{o}C and then freeze-dried, prior to being individually ground to a fine powder for component analysis. As in Chapter 3, DNA samples (n = 25) were used to develop a PCR-SSCP protocol for the 5'UTR, exons, introns and 3' UTR of dairy cattle *PLIN2*.

6.1.3. Gas Chromatography of the Fatty Acids in the Milk Sample

Gas Chromatography of the FAs in the milk samples was as described in Chapters 3.

6.1.3. PCR primers used for dairy cattle *PLIN2* amplification

Five sets of primers (Table 6.1) were designed to amplify target regions of *PLIN2*, based on the cattle reference sequence (RefSeq assembly accession: GCF_002263795.1). Region 1 spanned a portion of the upstream region (5' UTR) of *PLIN2*, exon 1 and part of intron 1. Region 2 spanned exon 2 (including the start codon), intron 2 and exon 3. Region 3 spanned part of intron 4, exon 5 and part of intron 5. Region 4 spanned part of intron 7 and part of exon 8 and Region 5 spanned part of exon 8 and part of intron 8 (Figure 6.3). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Table 6. 1 Primers used to amplify two regions of the dairy cattle perilipin-2 gene (*PLIN2*).

PLIN2 region[1]	Amplicon size (bp)	Forward primer	Reverse primer
1	589	5'-TGAATTACACGCAGATTC-3'	5'-CAAGAAATGAGAACCACGC-3'
2	398	5'-GAATCTTGTCACAGTGTTCT-3'	5'-GATCACTCTCAATGACTATAT-3'
3	446	5'-CCATGTTTCTCACCAGCCAG-3'	5'-GAAGAAGTTCCTTGGTGG-3'
4	483	5'-GCTGAATCCACTGCTCATTC-3'	5'-TTAGCTGCCTGCCTACTTCAG-3'
5	393	5'-CCAGATGACAGCTCCTCTTG-3'	5'-CCGATCTATTCTGCAGTGAA-3'

[1] **See Figure 6.3**

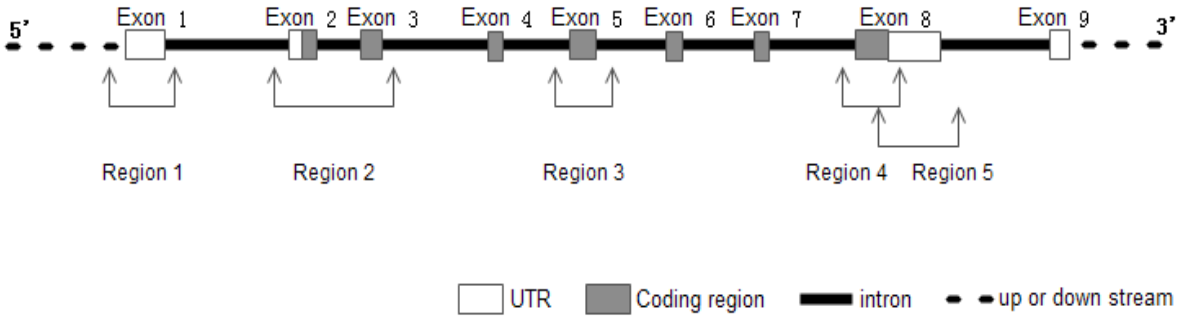


Figure 6. 3 Location of *PLIN2* regions that were amplified. Five sets of primers were designed in order to amplify a 589 bp region spanning the 5'UTR to intron 1; a 398 bp region spanning from intron 1 to intron 3; a 446 bp region spanning from intron 4 to intron 5; a 483 bp region spanning from intron 7 to exon 8; and a 393 bp region spanning from exon 8 to intron 8. The gene structure is based on the cattle *PLIN2* sequence and is not drawn to scale. Cattle *PLIN2* (RefSeq assembly accession: GCF_002263795.1) has 9 exons.

6.1.4. Developing the PCR-SSCP protocols for *PLIN2*

Dairy cattle DNA samples (n = 25) were used to develop a PCR-SSCP protocol for analysis of the target regions of *PLIN2*. The PCR protocols were optimised with different annealing temperature gradients (between 50 ^{o}C and 62 ^{o}C). Electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels was used to visualise the target amplicons, and 1× TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide was used during the electrophoresis. When the agarose gels produced a satisfactory result, the conditions for band separation and resolution were optimised at different percentage of acrylamide gels (10 %, 12 % and 14 %), and at various temperatures.

6.1.5. PCR amplification and SSCP analysis

PCR amplification were performed in a 15-µL reaction containing the genomic DNA (punch of FTA paper), 0.25 µM of each designed primer, 150 µM of each dNTP (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the polymerase enzyme.

Amplification was undertaken using S1000 thermal cyclers (Bio-Red, Hercules, CA, USA) and the thermal profile included an initial denaturation for 2 minutes at 94 ^{o}C; followed by 35 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 58 ^{o}C and 30 seconds at 72 ^{o}C; with a final extension for 5 minutes at 72 ^{o}C. Following amplification, a 0.7-µL aliquot of the PCR products was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ^{o}C for 5 minutes and rapid cooling on wet ice, the samples were loaded on 16 cm × 18 cm, acrylamide: bisacrylamide (37.5: 1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) in 0.5× TBE buffer. The method of Byun *et al.* (2009) was used to silver-stain the gels.

For Region 1, the primers didn't appear to work.

For Region 2, the optimised annealing temperature was 58 ^{o}C. The Region 2 amplicons were loaded onto 16 cm x 18 cm, 12% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels with 4% of glycerol and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 350 V for 19 hours at 26 ^{o}C in 0.5× TBE buffer.

For Region 3, the optimised annealing temperature was 58 ^{o}C. The Region 3 amplicons were loaded onto 16 cm x 18 cm, 12% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels with 4% of glycerol and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 390 V for 19 hours at 15 ^{o}C in 0.5× TBE buffer.

For Region 4, there was no variation found in this region.

For Region 5, the optimised annealing temperature was 58 ^{o}C. The Region 5 amplicons were loaded onto 16 cm x 18 cm, 12% acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels with 1% of glycerol and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 19 hours at 20 ^{o}C in 0.5× TBE buffer.

6.1.6. Sequencing of the dairy cattle *PLIN2* Region 2, 3 and 5 variants

Homozygous PCR amplicons identified using PCR-SSCP were sequenced at the Lincoln University DNA Sequencing Facility. The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment, translation and comparisons. The BLAST algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

6.1.7. Statistical analysis

Hardy-Weinberg equilibrium (HWE) for the *PLIN2* genotypes was analysed using an online chi-square calculator (http://www.oege.org/software/hwe-mr-calc.shtml).

All statistical analysis was carried out using IBM SPSS version 22 (IBM, NY, USA). Associations between variation in *PLIN2* and variation in milk FA traits were tested using General Linear Mixed-effects Models (GLMMs). As some measurements were made in percentages, a gamma regression function was adopted in the GLMMs. Single-variant presence/absence models (fixed effects: DIM, age and herd) were used to ascertain which variant should be analysed in subsequent multi-variant models. The multi-variant models included any variant that had a variant-FA trait association in the single-variant presence/absence analysis with a *P* - value of less than 0.200. The multi-variant models were again corrected for the fixed effects of (DIM, age and herd) and with variant fitted as a random effect. A GLMM (fixed effect: genotype, DIM, age and herd) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes with a frequency greater than 5% (thus insuring adequate sample size), on milk FA traits.

Interactions between different genes might be expected. To correct for the effects of other genes, another GLMM (fixed effect: genotype, DIM, age, herd, *DGAT1* p.K232A genotype, *FABP4* genotype and *SCD1* p.A293V genotype) and multiple pair-wise comparisons with Bonferroni corrections were used to ascertain the effect of genotypes with a frequency greater than 5% (thus insuring adequate sample size), on milk FA traits.

The effect of sire of cow could not be included in the GLMMs. Some semen straws (sire genetics) used in NZ dairy cattle artificial insemination breeding, contain mixed-sire semen purchased from commercial semen producers. In these cases, individual sire identity is impossible to ascertain, but because the straws were mixed-semen straws and because

different sires are used for different inseminations in different years, it is unlikely that sire was a strongly confounding effect. Cow age and herd might also be confounded with sire, but this cannot be confirmed.

6.2. Results

6.2.1. Variation in *PLIN2*

The PCR-SSCP banding patterns for different genotypes are illustrated in Figure 6.4a. Five nucleotide sequence variants were found in the three regions studied (Figure 6.4 b). The deletion variant c.595+104_595+108del described here (TGGCA/-) was at the same location as the previously reported deletion variant rs380629765 (CATGG/-). Three variants (A_2, B_2 and C_2) of Region 2, three variants (A_3, B_3 and C_3) of Region 3 and two variants (A_5 and B_5) of Region 5 were detected and then used to describe the associations between milk fat composition and variation in *PLIN2*.

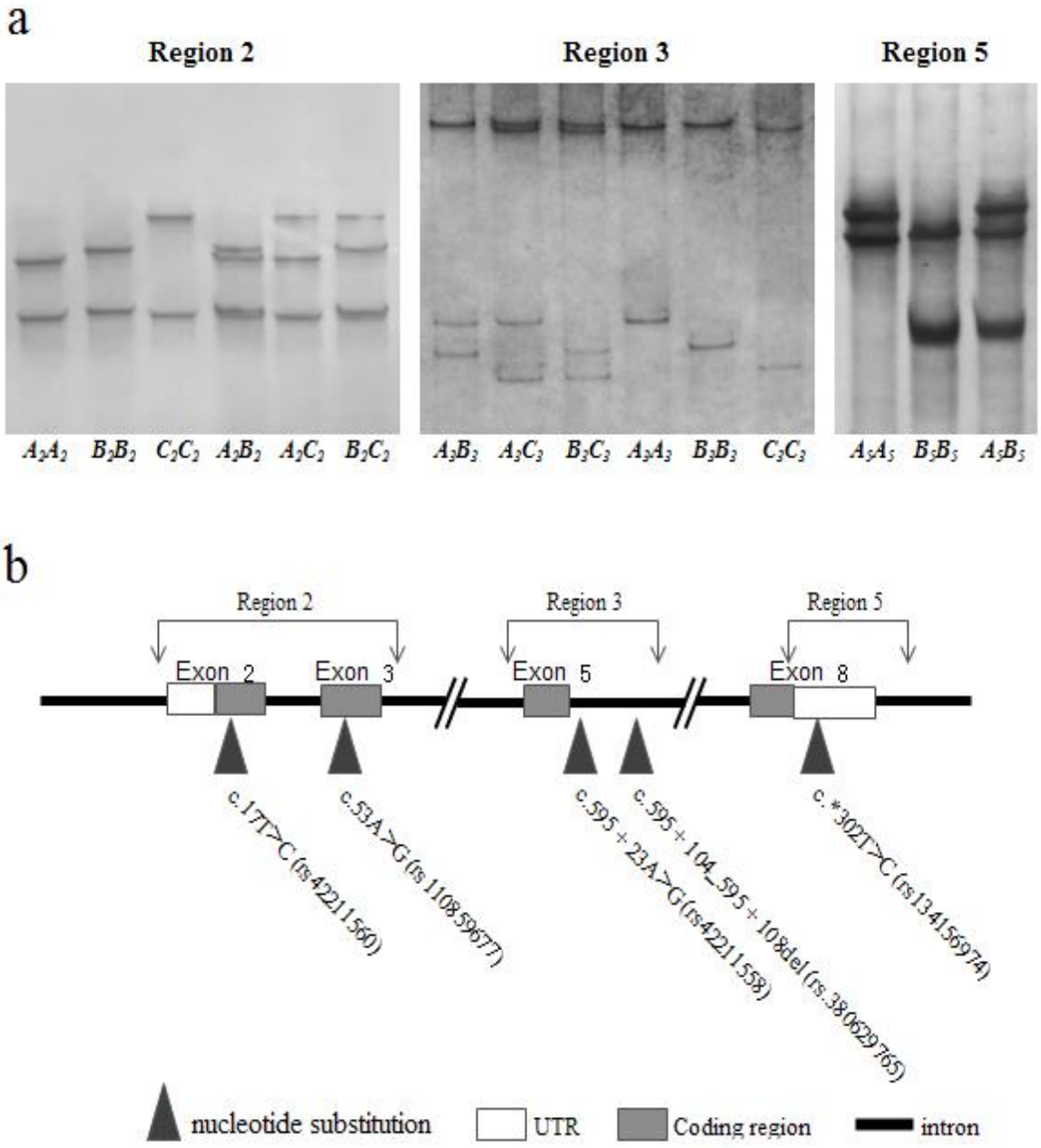


Figure 6. 4 Variation in the bovine *perilipin-2* gene (*PLIN2*). (a) Region 2 produced three PCR-SSCP banding patterns (A_2, B_2 and C_2) in the cattle studied, Region 3 produced three PCR-SSCP banding patterns (A_3, B_3 and C_3) and Region 5 produced two PCR-SSCP banding patterns (A_5 and B_5). (b) The five nucleotide substitutions identified were located in exon 2, exon 3, intron 5, and the 3'UTR. The gene structure is based on the cattle *PLIN2* sequence and is not drawn to scale (RefSeq assembly accession: GCF_002263795.1).

Six genotypes A_2A_2, B_2B_2, C_2C_2, A_2B_2, A_2C_2 *and* B_2C_2 were observed for Region 2, with frequencies of 43.0%, 13.4%, 0.3%, 34.8%, 5.7% and 2.7% respectively. The most common

variant was A_2 (63.3%) and the frequency of B_2 and C_2 were 32.2% and 4.5% respectively. The *P*-value for the chi-square for deviation from HWE was 0.044, suggesting the population was not at equilibrium.

Six genotypes A_3A_3, B_3B_3, C_3C_3, A_3B_3, A_3C_3 *and* B_3C_3 were found for Region 3, with frequencies of 32.3%, 32.0%, 16.4%, 10.3%, 6.8% and 2.2% respectively. The most common variant was A_3 (56.5%) and the frequency of B_3 and C_3 were 29.7% and 13.8% respectively. The *P*-value for the chi-square for deviation from HWE was 0.469, suggesting the population was at equilibrium.

Three genotypes A_5A_5, A_5B_5 *and* B_5B_5 were found in Region 5, with frequencies of 16.9%, 53.3% and 29.8% respectively. The most common variant was B_5 (56.5%) and the frequency of A_5 was 43.5%. The *P*-value for the chi-square for deviation from HWE was 0.089, suggesting the population was at equilibrium.

6.2.2. Milk traits, milk fat compositions and *PLIN2* variation

Associations between *PLIN2* variation in the amplified regions and gross milk traits (i.e. milk yield, milk fat percentage and milk protein percentage) were analysed in this study. No associations were observed between variation in either Region 2 or Region 3 and variation in these traits (Results not shown).

At the level of milk fat composition level, variation in milk FA profile was also not affected by the variation in Region 2 or 3 or was small (less than 5%), hence these results are also not shown.

In Region 5, three genotypes (A_5A_5, A_5B_5 and B_5B_5) were identified resulting from the nucleotide substitution c.*302T>C that is located in the 3'UTR of *PLIN2*. The associations between these genotypes and milk traits are listed in the Table 6.2. The effects of c.*302T>C on gross milk traits were not significant, but it was associated with variation in milk fat composition for C10:0, C11:0, C12:0, C13:0 and C16:0 levels, and MCFA levels. The B_5B_5 cows contained more C10:0, C11:0, C12:0, C13:0, and MCFA, but less C16:0 FA.

Table 6. 2 Association between milk FA levels and *PLIN2* genotypes identified in the amplified Region 5

Traits	Mean ± SE[1]			*P*
	A_5A_5 n = 69	A_5B_5 n = 218	B_5B_5 n = 122	
milk yield (L)	23.444 ± 0.574	23.563 ± 0.475	23.321 ± 0.533	0.819
milk fat (%)	5.007 ± 0.089	4.938 ± 0.074	4.950 ± 0.083	0.645
milk protein (%)	4.084 ± 0.048	4.033 ± 0.040	3.994 ± 0.045	0.117
C10:0 (g/100g milk FA)	3.141 ± 0.061^{b}	3.203 ± 0.050^{ab}	3.286 ± 0.057^{a}	**0.022**[2]
C11:0 (g/100g milk FA)	0.056 ± 0.003^{ab}	0.055 ± 0.002^{b}	0.060 ± 0.003^{a}	**0.022**
C12:0 (g/100g milk FA)	3.828 ± 0.081^{b}	3.887 ± 0.067^{b}	4.019 ± 0.076^{a}	**0.015**
C13:0 (g/100g milk FA)	0.114 ± 0.004^{ab}	0.113 ± 0.004^{b}	0.121 ± 0.004^{a}	**0.028**
C14:0 (g/100g milk FA)	12.488 ± 0.142	12.458 ± 0.118	12.658 ± 0.132	0.106
C16:0 (g/100g milk FA)	38.518 ± 0.507^{a}	37.742 ± 0.419^{ab}	37.388 ± 0.471^{b}	**0.048**
MCFA (g/100g milk FA)	20.621 ± 0.272	20.729 ± 0.225	21.155 ± 0.252	**0.033**
LCFA (g/100g milk FA)	49.540 ± 0.449^{a}	48.849 ± 0.371^{ab}	48.553 ± 0.417^{b}	0.052

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)' and 'herd' were fitted to the models as fixed effects. Means within a row that do not share a superscript letter are significantly different at $P < 0.05$.
[2] $P < 0.05$ in bold.

After correcting for possible interactions from *DGAT1*, *FABP4* or *SCD1*, the effects of the variation c.*302T>C remained for the C14:0 and LCFA level (Table 6.3).

Table 6. 3 Association between milk fat composition and *PLIN2* variant (c.*302T>C) *corrected for DGAT1, FABP4* and *SCD1 genotype.*

Traits (g/100g milk FA)	Mean ± SE [1] (g/100 g milk FA) associated with *LPIN1* genotypes [1]			*P* value			
	A_5A_5 n = 68	A_5B_5 n = 216	B_5B_5 n = 121	*DGAT1*	*FABP4*	*SCD1*	*PLIN2*
C10:0	3.167 ± 0.063^{b}	3.216 ± 0.054^{ab}	3.310 ± 0.059^{a}	0.235	0.149	**0.001**[2]	**0.018**
C11:0	0.057 ± 0.003^{ab}	0.055 ± 0.003^{b}	0.061 ± 0.003^{a}	**< 0.001**	0.067	**0.001**	**0.009**
C12:0	3.855 ± 0.085^{b}	3.897 ± 0.072^{b}	4.043 ± 0.080^{a}	0.298	0.121	0.061	**0.011**
C13:0	0.116 ± 0.004^{ab}	0.115 ± 0.004^{b}	0.123 ± 0.004^{a}	**< 0.001**	0.093	**0.001**	**0.013**
C14:0	12.616 ± 0.137^{ab}	12.516 ± 0.116^{b}	12.754 ± 0.128^{a}	**< 0.001**	**0.014**	**< 0.001**	**0.027**
C16:0	38.288 ± 0.502^{a}	37.505 ± 0.424^{ab}	37.075 ± 0.468^{b}	**< 0.001**	0.097	0.307	**0.021**
MCFA	20.809 ± 0.277^{ab}	20.813 ± 0.234^{b}	21.304 ± 0.259^{a}	**0.003**	**0.037**	**0.001**	**0.016**
LCFA	49.213 ± 0.435^{a}	48.546 ± 0.367^{ab}	48.174 ± 0.406^{b}	**< 0.001**	0.086	0.328	**0.023**

[1] Predicted means and standard error of those means derived from GLMM. 'Cow age', 'days in milk (DIM)', 'herd', 'DGAT1 p.K232A', 'FABP4' and 'SCD1 p.A293V' were fitted to the models as fixed effects. Means within a row that do not share a superscript letter are separated by Bonferroni test at $P < 0.05$.
[2] $P < 0.05$ in bold.

6.3. Discussion

Ogorevc *et al.* (2009) summarized the relationship between BTA 8 QTLs and milk traits (Figure 2.11), identifying that the region that contains *PLIN2* (*ADFP*) has QTLs associated with milking speed, protein percentage, somatic cell score, somatic cell count, and clinical mastitis occurrence. They did not find associations with other milk traits. In contrast, Lu *et al.*

(2016) found evidence that perilipin-2 levels were associated with variation in milk fat. Their mass spectrometry-based proteomics approach revealed that the concentration of perilipin-2 in bovine milk was higher in large fat globules (7.6 ± 0.9 μm), than in small ones (3.3 ± 1.2 μm). These large fat globules also contained more total SFA, C17:0 and C18:0 but less C10:1, C12:1, C14:1 *cis*-9, C18:1 *cis*-9 and CLA. Whether this is a consequence of sequence variation in the perilipin-2 gene was not tested.

The association reported here between *PLIN2* variation and milk fat composition may be because of variation in gene expression. The c.*302T>C nucleotide substitution is in the 3'UTR of the gene, and this region of eukaryote genes can contain regulatory elements that influence gene expression. For example, 3'UTR regions can contain microRNA response elements, AU-rich elements, iron response elements and other 'signatures' that can affect translation and mRNA stability. Sequence variation in these or similar regulatory elements might therefore change their function, and thus the level of gene expression.

For example, a nucleotide substitution c.*382A>G in the 3'UTR of the high-mobility group box protein 1 gene (*HMGB1*), alters the binding of bta-miR-223, and was found to be associated with somatic count scores in dairy cows (Li *et al.*, 2012). Similarly, Ju *et al.* (2018) revealed that the 3'UTR variation c.*301A>G (rs 211286607) in the neutrophil cytosolic factor 4 gene (*NCF4*) affects the binding of bta-miR-2426, and that cows with the GG genotype had a lower somatic cell score than cows with the AA genotype. Using a quantitative real-time PCR (RT-qPCR) assay, they also revealed that the cows with genotype GG had a higher expression of *NCF4* mRNA, compared to the cows with genotype AA.

Other researchers have also described 3'UTR variation in genes that affect milk traits. For example, in describing the effect of DGAT1 p.K232A, Grisart *et al.* (2002) described the 3'UTR variation c.*85T>C, but suggested this is 'more likely to be neutral'. Weikard *et al.* (2005) reported two nucleotide substitutions, c.*967C>A and c.*2922C>T, in the bovine peroxisome proliferator-activated receptor-gamma coactivator 1α gene (*PPARGC1A*) 3'UTR. They reported a trend that cows with the *PPARGC1A* c.*967C>A 3'UTR genotype AA contained a high milk fat yield (25.04 ± 4.29 kg) than cows with the CC genotype (16.77 ± 3.90 kg, $P = 0.076$). Khatib *et al.* (2006) reported associations between milk fat yield and the 3'UTR nucleotide substitution c.*223C>A (described as SNP 8232) in the oxidized low-density lipoprotein receptor gene (*OLR1*). They suggested that c.*223C>A might control the translation or stability of *OLR1* mRNA, because expression levels were lower in the AA genotype cows than the AC and CC cows. In the context of the above studies, it could be

concluded that variant c.*302T>C might affect *PLIN2* expression, but further studies will be needed to ascertain how that may be happening.

In the process of milk fat formation, perilipin-2 regulates the filling of milk lipid droplets with triglyceride. Both Thering *et al.* (2009) and Lu *et al.* (2016) reported that perilipin-2 appeared to affect LCFA transport, lipid sequestration and lipid storage. In this study, a significant association between *PLIN2* variation and a milk C16:0 levels were found (Table 6.2). Compared to the C16:0 result, the nucleotide substitution c.*223C>A appeared to have an opposite effect on MCFAs. For example, the B_5B_5 cows had more C10:0, C11:0, C12:0 and C13:0 in their milk (Table 6.2), although the results for the C11:0 and C13:0 levels are difficult to interpret as the homozygous B_5B_5 and heterozygous A_5B_5 cows differ at $P < 0.05$, but are not significantly different to the A_5A_5 cows. This is a rather confusing finding, although in both cases the levels of the FAs are very low, and likely close to the detection limits of the GC analysis. These enigmatic differences in C11:0 and C13:0 levels may therefore be a consequence of machine error and hence not real.

Bionaz *et al.* (2008b) described the gene networks in bovine milk fat synthesis (Figure 2.8). The C14:0 was mainly derived from *de novo* synthesise in the mammary gland. Except the *PLIN2*, the upstream genes might affected its component level. For example, the Cater 3, 4 and 5 found that *DGAT1*, *FABP4* and *SCD1* could affected the C14:0 levels significantly. After correcting for possible interactions with these genes, confusing results on C11:0, C13:0 and C14:0 levels were still existed (Table 6.3). Owing to the proportion of C14:0 in milk fat was not low, other *de novo* synthesise related genes might lead to the confusing results on C14:0 level, such as the FA synthase gene (*FASN*), Acetyl-coenzyme A carboxylase alpha gene (*ACACA*) and acyl-CoA synthetase short-chain family member 2 gene (*ACSS2*).

Chapter 7 Variation in *LPIN1* and its association with milk traits and milk fat composition

Our current understanding of the mammalian lipin proteins (lipin-1, lipin-2, and lipin-3) is that they are phosphatidate phosphatase (PAP) enzymes. They catalyse a key reaction in glycerolipid biosynthesis and the three isoforms are involved in the dephosphorylation of phosphatidic acid to form diacylglycerol. Overall, the Lipin family therefore catalyse a key step in the synthesis of TAG, phosphatidylcholine and phosphatidylethanolamine (Donkor *et al.*, 2007; Han *et al.*, 2006).

Phan *et al.* (2004) reported that the gene for lipin-1 (*LPIN1*) is associated with adipose tissue development and triglyceride accumulation, and lipin mRNA is expressed in a cow's mammary tissues during lactation, with the predominant lipin mRNA being from *LPIN1* (Bionaz *et al.*, 2008a). Variation in *LPIN1* has been reported to be associated with dairy traits. For example, variation c.2529+268C>A (rs137457402) in intron 19 of *LPIN1* is associated with milk protein percentage in Brown Swiss cows (Cecchinato *et al.*, 2014). This variation has also been reported by Pegolo *et al.* (2016), and associated with C18:2 *cis*-9, 12 levels in milk. Another variation investigated by Pegolo *et al.* (2016), was the variant c.6+10953G>A (rs136905033) in intron 1. It was associated with milk fat composition for C15:0, C14:1 *cis*-9, C16:1 *cis*-9 and C18:3 *cis*-9, 12, 15 levels.

The amino acid motifs 'DIDGT' and 'LXXIL' are important for lipin-1 PAP activity and transcriptional coactivator activity respectively (Finck *et al.*, 2006; Han *et al.*, 2006). In this study, genetic variability in exon 16 (contains the DIDGT motif) and exon 17 (contains the LXXIL motif) of bovine *LPIN1* were investigated to ascertain if they affected milk fat traits.

7.1. Materials and methods

7.1.1. Animals and milk sample collection

The Lincoln University Animal Ethics Committee (AEC Number 521) approved this research under the provisions of the Animal Welfare Act 1999 (NZ Government). The same cows described in the Chapters 3 were investigated here. A total of 450 Holstein-Friesian × Jersey (HF×J) - cross dairy cows were from two herds (124 cows in herd 1, 326 cows in herd 2). All the cows investigated were 3 to 10 years old and they were grazed on pasture (a mixture of

perennial ryegrass and white clover) on the Lincoln University Dairy Farm (LUDF; Canterbury, NZ). All the cows calved over the months August-September. They were milked twice a day throughout lactation (from calving until the end of May).

Blood samples were collected using the methods described in Chapters 3. The blood samples were analysed at the Lincoln University Gene-Marker Laboratory. A two-step washing procedure (Zhou *et al.*, 2006) was used to purify genomic DNA.

A milk sample was collected from each cow, in a single afternoon milking on 15th January 2014 (days in milk (DIM) = 148 ± 19 days). The milk samples were frozen at -20 ^{o}C and then freeze-dried, prior to being individually ground to a fine powder for component analysis. As in Chapter 3, DNA samples (n = 25) were used to develop a PCR-SSCP protocol for exon 16 and exon 17 of dairy cattle *LIPN1*.

7.1.2. Gas Chromatography of the Fatty Acids in the Milk Sample

Gas Chromatography of the FAs in the milk samples was as described in Chapters 3.

7.1.3. PCR primers used for dairy cattle *LPIN1* amplification

Two sets of primers (Table 7.1) were designed to amplify the targeted regions of *LPIN1*, based on the cattle reference sequence (RefSeq assembly accession: GCF_002263795.1). Region 1 spanned part of intron 15, exon 16 and part of intron 16, and Region 2 spanned part of intron 16, exon 17 and part of intron 17 (Figure 7.1). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Table 7. 1 Primers used to amplify two regions of the bovine LPIN1 gene (*LPIN1*).

LPIN1 region[1]	Amplicon size (bp)	Forward primer	Reverse primer
1	370	5'-TCCAGTGGACACAGAGCTC-3'	5'-CATCGTTGCTCAGTCTCAG-3'
2	368	5'-CATACTCTCTTCCATTGTG-3'	5'-GCTCCGCATTTGAATTCAGT-3'

[1] See Figure 7.1

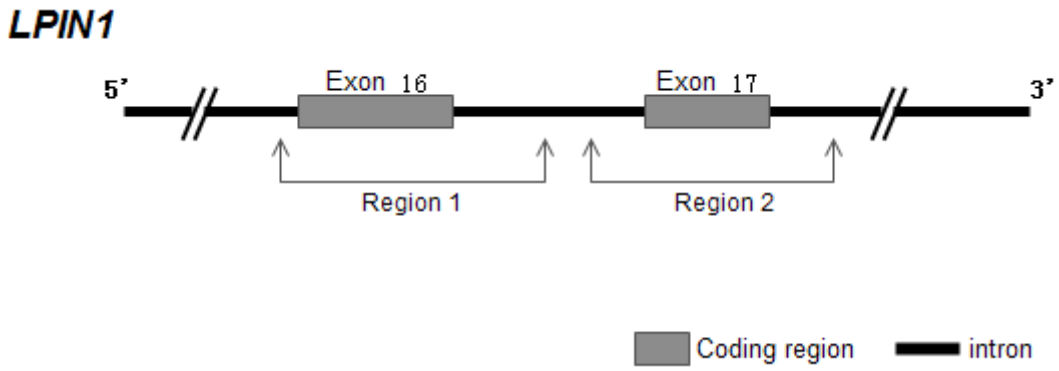


Figure 7. 1 Location of PCR primers designed to amplify five regions of bovine *LPIN1*. Region 1, a 370 bp region containing part of intron 15, exon 16 and part of intron 16; Region 2, a 368 bp region containing part of intron 16, exon 17 and part of intron 17. The gene structure is based on the cattle *LPIN1* sequence and is not drawn to scale (RefSeq assembly accession: GCF_002263795.1).

7.1.4. Developing the PCR-SSCP protocols for *LPIN1*

DNA samples (n = 25) were used to develop a PCR-SSCP protocol for analysis of the targeted regions of bovine *LPIN1*. The PCR protocols were optimised by testing different annealing temperature gradients (between 50 ^{o}C to 62 ^{o}C). Electrophoresis was undertaken in 1% agarose (Quantum Scientific, Queensland, Australia) gels containing 1× TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) and 200 ng/mL of ethidium bromide to visualise the amplicons. When the agarose gels produced a satisfactory result, the conditions for band separation and resolution were optimised in different percentage acrylamide gels (10 %, 12 % and 14 %), and at various temperatures.

7.1.5. PCR amplification and SSCP analysis

PCR amplification were performed in a 15-µL reaction containing the genomic DNA (punch of FTA paper), 0.25 µM of each designed primer, 150 µM of each dNTP (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany), and 1× the reaction buffer supplied with the polymerase enzyme.

Amplification was undertaken using S1000 thermal cyclers (Bio-Red, Hercules, CA, USA) and the thermal profile included an initial denaturation for 2 minutes at 94 ^{o}C; followed by 35 cycles of 30 seconds at 94 ^{o}C, 30 seconds at 58 ^{o}C and 30 seconds at 72 ^{o}C; with a final

extension for 5 minutes at 72 ^{o}C. Following amplification, a 0.7-µL aliquot of the PCR products was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ^{o}C for 5 minutes and rapid cooling on wet ice, the samples were loaded on 16 cm × 18 cm, acrylamide: bisacrylamide (37.5: 1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) in 0.5× TBE buffer. The method of Byun *et al.* (2009) was used to silver-stain the gels.

For Region 2, the primers didn't appear to work and no amplicons were produced under a wide variety of conditions.

7.2. Results

There was no variation detected in Region 1 (Results not presented).

7.3. Discussion

Finck *et al.* (2006) described how mutations in the 'DIDGT' and 'LXXIL' motif of *LPIN1* affected the peroxisome proliferator-activated receptor γ (PPAR γ) coactivator 1 α (PGC-1 α), which controls hepatic lipid metabolism in mice. However, the *LPIN1* sequences that encode these amino acids in Kiwicross^{TM} cows were conserved. This is possibly a function of the breeds and herds of cattle studied, with either prior selection and breeding, or some other breed or species-specific effect leading to the result that no variation in *LPIN1* was observed. A similar situation was observed for Kiwicross^{TM} cattle *DGAT1*, with there being no variation detected in regions 2, 3, 4, 6 and 7 (see Chapter 3). Further investigation of *LPIN1* in more breeds of cattle will therefore be required to determine if the previously reported variation in the 'DIDGT' and 'LXXIL' motifs in mice, are present in cattle.

Chapter 8 Summary

The genes *DGAT1*, *FABP4*, *SCD1*, *PLIN2* and *LPIN1* have all been reported to be involved in milk fat synthesis. This study investigated whether these genes were associated with gross milk traits and milk fat composition. Nucleotide sequence variation in the genes was investigated in regions that were thought to be of functional importance, such as the promoter, exon, intron and 3'UTR. Associations between DNA sequence variation and gross milk traits or milk fat composition were found for four of the genes in Kiwicross^{TM} cows. The association identified could contribute to the establishment of gene-markers that could improve dairy cattle breeding in New Zealand.

Variation in *DGAT1* leading to p.K232A was associated with variation in gross milk traits (volume, fat and protein percentage) and variation in milk fat composition in the Kiwicross^{TM} cows that were studied. The cows containing the K variant produced more milk fat and protein, but less milk volume. There were 18 individual FAs (these constituted approximately 65% of the FA in the milk fat) that were affected by p.K232A.

The gene *FABP4* was also investigated. The effect of *FABP4* variation on MCFA levels was different to what has been reported in previous studies, especially in respect to the levels of C8:0, C10:0, C12:0, and C14:0. This is also the first report of associations between *FABP4* variation and variation in LCFA levels, such as for C18:2 *cis*-9, 12, C22:0, C24:0, and C22:5.

The widely reported variation c.878C>T of *SCD1* was identified here and was associated with variation in MUFA levels. There was linkage between c.702A>G, c.762T>C, c.878C>T, c.880 + 105A>G, c.*1883C>T and c.*1984G>A, which are located in the exon 5, intron 5 and 3'UTR regions. This is the first report of associations between c.*1783A>G and c.*2066T>C, and milk fat composition in Kiwicross^{TM} cows. For example, variants *b* and *c* of *SCD1* affected C16:1, C17:1, C18:2 *cis*-9, *trans*-13, C18:2 *cis*-9, *trans*-12 and C16:1 index levels.

Variation in the 3'UTR of *PLIN2* was associated with many milk FA levels. The effects of *PLIN2* were mainly on the C10 – C16 FAs, with the 3'UTR variant c.*302T>C possibly affecting *PLIN2* expression and leading to changes in milk fat composition.

The DNA sequences of *LPIN1* investigated here were conserved in Kiwicross^{TM} cows. There was no variation detected.

Appendix A: Sequences of the *DGAT1* fragments amplified

Region 1:

ttcgctgtgaccctggcagcacctcaatcaggatccagaggtaccagggctgtaggccccgccctccccggaggccccgccctcccc
ggaggccccgccctccccggaggccccgccctccccggaggccccgccctccccggaggccccgccctgtatcaaccttggaccc
cgtcttcctcaaacaggccccgccccgccttggtacagaggcccttcctgattggtgccttcacagtccgtgccttctcattggcttgagg
ccctgatctctcaactccagcggtggaacccttggttccctcacgtcccgggtcagatcggttctctttgatgaccctcggcccaccctgg
tgtcctcactccagctgtttcatgttagccgaaggcaaaggagcctgga

Region 2:

tgcatttgccaggagaccacaactcccagggtgcaccgcgcgccagcggactacaaaggtatgcgcgccgcggccctgggccagtt
agctgctccgggaactacgcttcccaggactccgagaggagccgtccggcacggatttgcacgcgctgattggcggcgcggaccac
ggcagtggcgtagtagaggcg

Region 3:

ctcaacttctagacgccctcctctgccttcctttggtgggttctgaagctttcccagggtgagcccactacgcacagtgtcctctacctgg
aaggagatacaggggtccttcctgagggctatgaggggtgccttgtgggttgataaagctcccgggggaggagggtggaccggcgg
agaacagaggcaggggcagtgcgaggggatttctcatccctcgcagaccctccagagaatggtcttcacaaaggtccctcatccgtca
cccggcgattgactggcctaggatcctgcttattaccagcacaaatggctgctctagggtcaaagtgggtcctgtaatgggaccctcac
ccctggttggggtacaggggaggagttggaagtgcgcacacccacaggtgggcgccctgcttagctgaaggactgatg

Region 4:

cacaggtgagtggtcttgggggtccacgtagaacttcctcttctgttccaaattgccctcatgggtgcggcatgcctgggtgaacctggg
ggagcagggtgaggacatgcttctcagcccagcccacagctccaggccacactctgcaggactctggcccctccctcagccctgga
gggagcaggactggagtcctgtgtccgccttgctctgacctggccgaggccactgctgtggggccccagcaggcctgcccagcaga
aggtggagtgcagggaccccaggggcagccttcagggtggggcagggtgaggcccgactgggcccagccccaccgctcagtgct
gatgtggcgcgaggccttcgccctccagctgacgtgtctg

Region 5:

ccactgggctgccacttgcctcgggaccggcaggggctcggctcacccccgacccgccccctgccgcttgctcgtagctttggcagg
taag**[GC/AA]**ggccaacgggggagctgcccagcgcaccgtgagctaccccgacaacctgacctaccgcggtgaggatcctgc
cgggggctgggggggactgcccggcggcctggcctgctagccccgccctcccttccagatctctactacttcctcttcgcccccaccct
gtgctacgagctcaacttcccccgctccccccgcatccgaaagcgcttcctgctgcggcgactcctggagatggtgaggcggggcct
cgcgggccagggtgggcgggcctgccggcacccggcaccggggctcagctcactgtccgcttgcttc

Region 6:

ccccggcaccggggctcagctcactgtccgcttgcttccttccccagctgttcctcacccagctccaggtggggctgatccagcaggta
cgtgcccgggggggggggggggggggggggggggggacggggactctggggccgttggggagctgactctgcgctttttgcagtg
gatggtcccggccatccagaactccatgaagcccttcaaggtgagcaggcaggcctggcagggtgggttccggggtcagggctgag
ggagccagctgtgccctgtgcccacaggacatggactactcccgcatcgtggagcgcctcctgaagctggcggtgagtggcctgctg
ggtggggacgtgtgggggcgggtggggctgttctggcacctggcacccactccccacaggtccccaaccacctcatctggctcatctt
cttctactggctcttccactcctgcctgaacgccgtggctgagctcatgcagtttggagaccgcgagttctaccgggactggtggtgggt
ggccttgccggggcgggggtggtgggggccccgctggggctggggccggagcccctgcccactctgc

Region 7:

tggtggtgggtggccttgccggggcgggggtggtgggggcccccgctggggctggggccggagcccctgcccactctgccccgc
ccccgcaggaactccgagtccatcacctacttctggcagaactggaacatccctgttcacaagtggtgcatcaggtgggtgtgcgcctg
ggggcgggggttgggggtgggacggggtcgcgtggcccggcgcccagcccactgccgcctcccccgcagacacttctacaag
cccatgctccggcggggcagcagcaagtgggcagccaggacggcagtgtttctggcctccgccttcttccacgaggtcagtgcactg
agggcgcgccctgcccctggtgggggtgggggtgggggtgggggctcgctgacgcccctctcccctcagtacctggtgagcatccc
cctgcgcatgttccgcctctgggccttcaccggcatgatggcgcaggtgagcagccctggacccccgctccgccccgcccgcgag
cgcagaggctcactc

Region 8:

ctgtgcacagtgagctccctcagcctccagggcacagggctggcaggaggggcggccctcccacgtggggccatgctgtgggaa
ggaggccccagcgcctggagaggagctggggctgtggtgaccctccctgcctcacagggctctgtggtcagacgtcttgccctgcaa
ggtggagactccatgctccaaggccccctgtgcctgaggtctgcacacaagtggattcaacttgggtcaggccagaggctaaggtgtg
gaagagggttgagaatcaggctgacttgaac

The letters with underline represent the primer coverage. The upper-case letters represent the nucleotide substitutions revealed in this study.

Appendix B: Sequences of the *FABP4* fragments amplified

Region 1:

atcatgagttccttattgccaaattcagacttaaattgaagaaagcagggaaaaccactagaccattcaggcatgacctaaatcaaaaca
acaacaacatgtcattatattaatactttgtccaaacacatggaacatataataccaaaagtgaaccctaaggtaatatatggactttgagtg
attataatataccaatataggttcattcttagtaaaaattaccattctggtgaatgatattgataatgggggaggctatccatatgcgggaac
agggagtacatgagaaatctctgtactttcctctcaattttgttataaacctgaaacttctctaaaaaattatcataaaagcaaaacaaactaa
aaacaaaagccatttattagtttagaacatgaagttatccctgtatgaaacttgatcatttttatttttcttgttcgatgaattcataatagtaagcc
taccctgaatggtacatcacccacagtctataatatgtctatg

Region 2:

gatgaattcataatagtaagcctaccctgaatggtacatcacccacagtctataatatgtctatgcaacagttcagactgaatgatcattatc
actgtgccgttaacatatgcaaccattaaaagttactattgtaacacaactttacccgtttttcttagcaaatttcatgcctacagaaaatctgt
cctttacggaatcctcttactcaaccatttctgggaagcccttaggattcctacaaatcaatgatatgctcagcagatacagttcagtgttata
cgcagtgcagtgatacggaacacaccccatgatgttttaatttttttttttctttcctaaaatctaggtgtgttcacgggagtttgttgcccatatg
accgtcttccatcattctggtggggctgcgggggggaccgataaacatggttactttagaaaagcattctcatgacgtatttgctatgtaaaa
gaagggaattagacctcagaagcttgtgactaccccactccagattgcatttaagtaaagcagcaccatttagatctggataaatttagag
cttataatataggtcaggaacaggacgaat

Region 3:

tagacctcagaagcttgtgactaccccactccagattgcatttaagtaaagcagcaccatttagatctggataaatttagagcttataatata
ggtcaggaacaggacgaattttctcagtaaggggagtatattttggatcggtgccttttttttaggaagtctgtggaaatccacactatgctc
ttatttagggaatggaaagaatgaggttaaatgatcatttgtcagttaaaactgctgtctagattcctggtggagaattaaacttacttttttctttt
ttcattcatataaaacatttgaaatgaggaagctgggatactttaaaatgccattacttattttgttttaatttccaggtaattcctgagacagta
ctgcccccaatagcctttgcaattacttaagaatatccaaggtaattcttacattcctcaattcaaacgaaccacataattacaattttaatag
gaactctagaaaagggagtcagaatttatcccaagataattacactgggtccactctacactggaataaata

Region 4:

acactgggtccactctacactggaataaatatgtataaaaaaaaaataggaaattcaatgcactgaattttaagctgtcaaaacaagattatt
gaaatattctgttaaaggttaaaaataagttgtactctgagtccagtgaccatttgccaaggagagccaaagttgagaaatttctattaaaa
acatgactcagagggaaaactgcagaggctggcaatgaaggaaatgatcggatatcattcccaattggttatgcccaagatcacatgat
ctgggcacctttaaaaggaagatatctggactcagagggtaatagcatcttgctgaaagctgcacttctttctcaccttgaagaattctaga
aagctcacaaaatgtgtgatgcatttgtaggtacctggaaacttgtctccagtgaaaactttgatgattacatgaaagaagtgggtaagga
aatgcattgttgaatggctgggcttataacttttctctaggaaagagaggcctatggttcttttcactctggaagcattggtctg

Appendix C: Sequences of the *SCD1* fragments amplified

Region 1:

aatcaggtaggtctcagcgtcccctctccactcactgagcctgtgatctctcaatgcaggtactacaaacctggtgtcctgttgttgtgcttc
atcctgcccacactcgtgcc**[A/G]**tggtatctgtgggatgaaacgtttcaaaacagcctgtttttgccaccttattccgtta**[T/C]**gcc
cttgggctcaacgtcacctggctggtgaatagtgctgcccatatgtatggataccgcccttatgacaagaccatcaacccccgagagaa
tattctggtttccctgggagctg**[C/T]**gggtaagtcagcagtccacagcaagaccacgtctagtggtctgctgcttagggtattaggtt
acgtgccagaaaaactagatttacctgttttatgacccctctcc**[A/G]**tatgtcattccactataaaactaagggacagtattagaa

Region 2:

gaaccactgtttctctttacaaagttgagcaagctgccactttcacttggcctccagagtctcc**[A/G]**tctatatccttgtgctccttacca
cactgatgactccagacaaggctggcaaagcctgctagaaacatcctgggcacaggcattcgcactcatgaggca**[C/T]**ggccaa
gccgaatgctcatgttgtgccagagccagccatggagcaaaagaggatttgtttttagtctcctctgtctgggtcagaaccagagagcat
gctg**[G/A]**atgcccccggcttactggataagctgcctaccctgagtcagtgctcccagcggacagtgcgaggcttgcagaagcag
gggg**[T/G/C]**gcctagccttcactgggaagcacaagaagcaaaggcaggttccaaagtg

Appendix D: Sequences of the *PLIN2* fragments amplified

Region 1:

tgaattacacgcagattctttaatgctgagccaccggggccccacttccttttgcgaaactacaaagccagcgtgggcacccgccgcat
atttgcacggggatccccagcactcttgatccaggcagggccgccccctacgcccgggcagaaggccgggggtcacgtggcacgg
gggcgggcaggggcggggcgggtatatattccggcgcgccggtcggtgagctgtgaacgcctcacagactcagcggatcttcgcg
cttgggcgtctgctggctggtgggtgcgcgcccctctgcggtccctgtggctcggccaacgcgggcttcgggccggacgctcgcgg
cctcgcggcggtttcggggccccggctgctgggaaggggcggtgtctggggcctgcgtttccaccctcgcttttgttcttggtcgaggc
ttgcttttctgctcgctgtcttggtgcgtttgtagctgcttggttgggttggggctagcagtttccaatcgaccccagacgacattagttaaaa
ggaaacgcgtgagaaaatcgtcttaaagctgaattttgtggggtacgcggacgggctaatgataactgttgcgccttcggcgtggttctc
atttcttg

Region 2:

gaatcttgtcacagtgttctgaaataagcttctcttctgcagatttcttctttctccagcaagaaagatggcatctgttgcag**[T/C]**tgaac
cacaactggtatgatttataaatagacggatgattcttgattccttttccccaccagccctgttttgtagacattaactcctccctccctcttggtt
gcagagtgtggtgaccagggtggcca**[A/G]**cctccccttggtgagctccacgtatgatctggtctcctcggcttacatcagtagaaa
ggatcagtatccttacttgaagtctctgtgtgagatggcagagaagggcatgaagaccatcacttctgtggctgtgaccagcgcgctgc
caatcatccaaaagttagagccacaaagtgagttttcattttggcctcaaaatttactcccatatagtcattgagagtgatc

Region 3:

ccatgtttctcaccagccagatggcctgctttttttccccataggttgtggccaatgccaaaggggctatgactggggcaaaagatgctgt
gacgactactgtgaccggggccaaggattctgtggccagcacaatcacgggggtggtggacaggaccaagggagctgtgactggta
gtgtggagaagaccaagtccgtggtcagtggcagcattaacacagtcctacgaagtcgggtgatgcagctgatgagcagtggagtag
aaaatgcactcaccaaatcagagctgctggtagaccagtacctccctcttaccaaggatgaactaggtaattggatttttgccttgaa**[A/
G]**taatgtgtttctttccttttttgccacgccatgctgcttatgggatttcagttccctgaccaagaattgaacccgggcca**[TGGCA/-
]**gtgtaagtccagaaccctgactactaggccaccaaggaacttcttc

Region 4:

gctgaatccactgctcattcttaaacttgtcttactttcagcacattgagtcacgtactcttgctattgcccggaacctgactcagcagctcca
gaccatgtgccacaccctcctgtccaacatccaggggttaccacagaacattcaagatcgggccaaccacttgggggtgatggctggt
gacatctactctgtgtttcgcaatgctgcctcctttaaagaagtgtctgatggcctcctcgcttccagcaaggggcagctgcagaaaatga
aggagtctttagatgatgtgatggattatcttgttaacaacacacccctcaactggctggtaggtccctttttatccccaggtgaccgagtct
gagagtgctcaggccccaggtacaaccaggaggcctggcaggtggagtagaaaacaccctaaacccgtccctgtcagcaatgcaga
gggcagccagccagatgacagctcctcttgagctgacacctgctagacaggtctgaagtaggcaggcagctaa

Region 5:

ccagatgacagctcctcttgagctgacacctgctagacaggtctgaagtaggcaggcagctaatgaggcaaaaaagtctccacttcagt
catttccaactggcataagagctatgaagtttctgcattagtgcataagttctgttacttacctggctggaaaaaaaagaataatagagtggt
cagagcctgtccagaactcgcacatgtttcagggtgtttatggcctcatgcttttgctacttactgttgctgtctgtctgtactgaataaaaac
accttcatgtaggctgttgtatgaattggggtctgctctgagcagg**[C/T]**ctcaactctggtttgtctcacaatgcatgcttgtgtaccctg
tgtttttatctttcataaagaagtgcctcctttgaattcaataaaattcactgcagaatagatcgg

Appendix E: Sequences of the *LPIN1* fragments amplified

Region 1:

tccagtggacacagagctcctgtctttcagaaaagcctgaagttgaagaacggcccgaacgacgtggtgttcagtgtcaccacgcagt
accagggcacgtgccgctgcgaaggcaccatctacttgtggaactgggacgacaaggtggtcatctctgacattgatgggaccatcac
taggtaagtctgagtccacctactctggggctgtgggccctgggcctggaggtgcgttggctcctcttgctggggccgattggggctca
tgtagttcagtttctggggctccttgaattaaacagctgcagtgggggcttggggatctcttccctctggggcagtggctccctgacatcg
ttgctcagtctcag

Region 2:

catactctcttccattgtggtttgtcacagggtatccactgtagttccctgtgctagacagtaggcactgcagttcacgcagtgccctcgtc
atttaatttttctgttctctgtctttattcagatcagatactctcgggcacattttgcccaccctggggaaggattggacccaccagggcatcg
ccaagctgtaccataaagtgagccagtaagtccacagctggggaacatgcggcagtctgcacacgtaacctttcttgctggtgtggctt
acacagacaggcgcaccctcagccagggagagttggcgggggtggtggtggagcacacggtggaagcctgccagctccgcatttg
aattcagt

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